

How to get back from the dead

Two developments in the British academic scene offer a remote chance that British universities may yet be revived. But there is no time for leisured consideration of what to do.

THE question whether the British university system will be able, like Lazarus, to rise from the dead will not be settled by the announcement last week that academic salaries are at last to be increased (see p. 567) or by the recommendations of the government committee under Lord Croham which, for the past year, has been brooding on the organization of the University Grants Committee (UGC), the conduit for the government's direct support of the universities. (The committee's report will have been published before this issue of *Nature*, but the essence of its proposals has been well flagged in advance.) More pay may persuade some able academics to postpone decisions either to emigrate or to look for more rewarding jobs in other sectors of the British economy. In the long run, there may be more hope in the proposal that UGC should be organized so that its chairman is an independent part-time person who is not an academic. But jaundiced bystanders will rightly suspend judgement about the long-term benefits of these developments, the second of which has not yet been accepted by the government.

British universities, and the system of which they are a part, have been put into a dream-like environment by the events of the past decade. It has been a curiously British process which began ten years ago, before the present government came to office, when the then government sought help from academics in containing the costs of student support and in planning for the demographic prospect of a declining population of young people (now a reality). It is understandable but not forgivable that, after a period of rapid publicly supported expansion, the university system should have hotly rejected the notion that budgets might be constrained. What those responsible appear not to have appreciated is that the government was then, as it still is, the paymaster.

Economies

Since the election in 1979, that proposition has been self-evident to all. The process of making the public cost of the university system shrink has nevertheless been accompanied by such a spate of uneconomic economies that there is no particular reason why those who work in the system should believe that the money that keeps their institutions alive is of the same currency as that the government uses to pay a weekly dole to more than three million people who are unemployed. Many universities and university departments have been prevented from taking as many students as they could usefully have taught. Some, considered to be too small when standing on their own, have been compelled by outside pressure to merge with others only to discover that student demand is stagnant. Many academics have been invited to leave early on generous terms, and have then been reemployed as part-time teachers. In the current academic year, most British universities are outspending their incomes in the fond, but unfounded, belief that some future government or UGC will bail them out. In a climate in which vacant posts are left unfilled for as long as possible, is it any wonder that younger academics prefer to work elsewhere, where opportunities for doing things for the first time persist?

British universities underestimate the damage being done to their well-being by the inclination of ambitious academics to

move elsewhere. Part of the trouble is that the numbers are not absolutely large — perhaps only a few hundreds each year. Most often, these people pack their bags and go elsewhere only when their promise has been recognized by other institutions able to offer posts promising facilities for research, some security and much opportunity. The direct consequence is that some of the more promising research projects in British universities are transferred elsewhere, but the indirect effects may be even more damaging. Who can suppose that the spirits of those left behind remain as high as they should be? And who can be surprised that now, after a decade of attrition, British universities are hard-pressed to recruit able graduate students from their undergraduates and able postdoctoral people from among their PhDs? One way or another, the academic profession in Britain must head off the danger that it will become a scorned profession.

Prospects

Last week's salary settlement will help a little, but not permanently. British academic salaries will even now not compete with those to be had in comparable occupations. And while it remains the case, as politicians (in Britain, even more poorly paid) are never tired of pointing out, that academic freedom and even vacations are worth a great deal, academics are acutely conscious that these intangibles will not pay grocery bills. In any case, individuals' immediate benefit will be offset by the way in which their institutions will have to dig deeper into their red ink to pay the extra, while the prospect that the government will not for the time being contribute to the extra cost after the first three years can only make financial planning seem even more an exercise in the unreal. The only potential long-term benefit of the settlement is that the salaries that universities may pay to individual professors will not in future be constrained. That, potentially, is a means by which some universities might keep some able academics on their books. They will pay for that freedom by the requirement that the performance of less senior people should be more often, and more explicitly, assessed, which will be no bad thing.

But will universities be able to take advantage of this freedom offered by the new pay settlement? Three pre-conditions will have to be satisfied. First, universities will have to devise some means collectively of making wise decisions about the quality of outstanding colleagues; their general indecisiveness on issues of the quality of research in the past few years does not promise well. Second, UGC (whether or not remodelled) will have to be prepared to deal generously with the better universities, embarking on the pursuit of excellence without shame. Third, and even further ahead, all parties to these bargains will have to acknowledge that by helping universities make bargains with individual academics, they are assisting in a process whose logical conclusion is a degree of autonomy that British universities do not now enjoy.

This is where the remodelling of UGC is indispensable. Under the present system, UGC tends towards equitability between institutions. Although it has begun on the process of distinguishing between institutions on the basis of their reputations in research, its first essays in this direction have been modest and



uncertain, while the Secretary of State for Education and Science has made it plain that he would not allow the process of discrimination to go so far as to threaten the existence of weaker institutions. The potential benefit of a UGC reorganized as a council on Croham lines is that it might be willing to take hard decisions of this kind, fighting the government for its right to be discriminatory between the good and the less good if that is what the circumstances require. The obvious danger is that such a council might shoot off in an eccentric direction, the pursuit of what is fashionably called 'relevance', not excellence, for example. On balance, the risks are worth running for the chance that greater autonomy will emerge.

Overlap

None of this will absolve the British government from worrying about higher education. For twenty years there has been an anomalous division of higher education into two sectors, one called the 'private' sector which is supported directly by central government through UGC and one called the 'public' sector because it is supported by central government through local authorities. Especially if universities are compelled by financial pressures to become more diverse in what they do (which would be welcome), and if Mr Kenneth Baker, the Secretary of State for Education and Science, succeeds in detaching the institutions called polytechnics (a large part of the public sector) from the local authorities, there will be even more overlap between the systems than there is at present. Will it then make sense to let them coexist as ostensibly different kinds of institutions? That is one issue to be settled soon. Another is whether it makes sense that UGC should continue to be the conduit for a notional £600 million a year intended for the support of academic research when the research councils, formally responsible for the same causes, are increasingly incapable of supporting projects which, by lapsing, help to drive the iron deeper into the heart of British academic science. Why should not the government settle these issues while deciding how to respond to Croham? That way, there might be a chance of returning the system to good health. □

Choose your timescale

US researchers, under pressure to be more productive, may find fewer benefits in plurality.

ALL governments believe that research, and particularly scientific research, is potentially a prolific source of wealth and, perhaps even more important, of international advantage. Even academic researchers know that. Most governments have also followed their declared precepts in arranging that research should be conducted in an orderly fashion. The most successful framework so far devised is that still in being in the United States, where the bulk of what is spent on what researchers do is spent by agencies which pretend they have a practical interest in the outcome. In reality, of course, the need for the US Department of Energy to know whether there is a *top* quark, a question that may be settled when its next or next-but-one accelerator is working, is no more compelling than the need that NASA should have a more rounded understanding of why the Universe expands. The framework succeeds only because the sponsors are tolerant of their pensioners' enthusiasms. In places such as Japan, where government agencies are required to be less flighty, public sponsorship of research is less catholic and also less productive.

For how long, in circumstances like these, can the casually creative system in the United States survive? There are both pluses and minuses. This week's report (see p. 564) that the US Navy cannot afford the \$400 million-odd that would have been required to build a better sea-surface satellite monitor is a far cry from the days (in the 1950s) when the US Office of Naval Research was investing in molecular biology on the grounds that

nobody could deny the possibility that aircraft — US Navy aircraft, of course — would one day be flown by appropriately coded nucleic acid molecules. Studies of the behaviour of porpoises were at the same time encouraged on equally free-thinking grounds. It is true that liberality of this kind has always gone hand-in-hand with even more generously supported projects of a strictly practical character, but inter-agency competitiveness has been a constant spur to farsightedness. That is another reason why the US Department of Energy's willingness to take the next big particle accelerator under its wing is to be welcomed; the project may do little for the state of the domestic oil-producing states, but it will ensure that some other agency (the National Science Foundation, perhaps?) does not get hold of it. Researchers know well enough the benefits of this lavish plurality.

That is why the events of the past few weeks in the United States are mildly disturbing. The new budget, at the beginning of the year, is above all tidy. The National Science Foundation (NSF) is destined to grow. Other agencies, such as the National Aeronautics and Space Administration (NASA), will get more than last year if only Congress agrees, but will have more of the money tied up in specific tasks (building another shuttle spacecraft, for example). It could easily be that the pluses, especially the more open recognition that NSF should prosper so as to support more and better research, will be offset by the minuses arising from the workaday mould into which the mission-oriented agencies are being forced by the budget problems of the United States. It is far too soon to begin worrying that the condition of research in the United States will soon resemble that in, say, Britain, for the margins for free spending by US agencies are still very broad. But there are unwelcome signals in the single-mindedness of the mission-oriented agencies that could yet force US researchers to follow the short-term conventions common elsewhere. Ironically, if the worst should happen, the damage will have been done in the name of good government. □

What does ABM mean?

The prospect of a transatlantic quarrel over the Anti-Ballistic Missile Treaty cannot be avoided.

IT appears that the US administration is drifting towards serious disagreement with Western Europe over the deployment of some elements of the Strategic Defense Initiative (SDI). The difficulty is the wish of many in and just outside the administration to have something of SDI in place before the end of President Reagan's term in office, roughly 22 months from now (see *Nature* 325, 470; 1987). The wish is easily understood, even by those who do not share it. The puzzle (to Western Europeans) is that the United States seems not to understand, let alone to have anticipated, why the issue is so important that even the secretary-general of the North Atlantic Treaty Organization, Lord Carrington, should have written to Washington for an explanation of what is going on.

The origins of the Anti-Ballistic Missile (ABM) Treaty of 1972 refer, then, at the beginning of what turned out to be 70 per cent of a decade of detente, the United States accepted that the Soviet Union had the capacity to defend a few targets (Moscow in particular) against some missiles, that the cost and difficulty of its own development of a high-acceleration missile called SPRINT would not be worth the candle and that there was strength in the general opinion that, to the extent that ABM defences must undermine the now-traditional theories of deterrence by the threat of mutually assured destruction, they are best avoided. President Reagan made a good debating point in March 1983 when he argued that it must be preferable to prepare to defend one's people against attack than to prepare to attack potential attackers. But the issue has never been debated. That is why, late in the day, there must now be an argument. □

Europe alarmed at US plan for space developments

- SDI could break ABM treaty
- Space station — a military base?

Washington & London

EUROPEAN governments are increasingly concerned by what they see as the drive by the US military to dominate space through the Strategic Defense Initiative (SDI) as well as by a renewed interest in the space station programme.

Mr George Schulz, the US Secretary of State, has met European delegates during the past week in an attempt to allay their fears over what appears to be a new political momentum behind an early deployment of SDI. And representatives of the European Space Agency (ESA) have been in Washington trying to determine the motives behind the plans being developed by the Department of Defense (DoD) for the space station.

The Belgian foreign minister and present head of the Council of Ministers of the European Communities, Mr Leo Tindemans, claimed that there is extensive unease among European governments about SDI. The Anti-Ballistic Missile (ABM) Treaty, now 15 years old, restricts full deployment, the European governments claim, and they want research on the SDI project to be curtailed to comply. The United States appears not to agree.

But the Europeans are more than surprised at the revived interest in the space station, previously considered an exclusively civil project. The SDI ambitions of the United States and the new plans for the station, many European observers fear, could exert pressure on the North Atlantic Treaty Organization and scupper any hopes of early agreement with the Soviet Union on restricting the use of nuclear weapons. The Soviet Union has consistently tried to arrest the progress of SDI and considers the project to be substantially less advanced than is claimed by the United States.

European participation in the US project to build a space station will depend on a meeting arranged for 11 and 12 February in Washington with all international participants. An ESA delegation will examine several issues raised in a document prepared by NASA after discussion with US government agencies on the future military interests in the space station.

Negotiations planned for last month were delayed at the request of DoD to give it time to review its interest in participating in the multinational project.

The new US bargaining position was worked out late on the evening of 4 February, and transmitted to the countries in-

involved. Although no details of the new US position were released, NASA administrator James Fletcher told a House of Representatives committee on Thursday that the space station would still be reserved for peaceful purposes.

In other testimony, Fletcher maintained that the administration's request for \$767 million for 1988 for the space station would meet the goal of operational capability "in the mid-1990s". But it is certain that the current \$8,000 million estimated cost for the space station will go up. NASA has completed two separate analyses of space station costs following the completion of the preliminary design and definition phase of the project. The details are being worked out with the White House Office of Management and Budget, but the cost is likely to rise as high as \$13,000 million, if not higher.

A spokesman for ESA said last week that the purpose of this week's meeting is less to negotiate the US draft than to study the practical consequences of what NASA now proposes should be the role of the US Department of Defense in the design, management and operation of the space station. All the member governments will be represented on the ESA delegation by at least one member. The political implications of the draft document will be decided only when the delegation has returned from the United States. It will then be open to ESA governments to seek changes in the draft plan.

ESA hopes that it will still be possible to agree by spring on the development of its Columbus project as an adjunct to the space station proper. But the timetable has been thrown awry by DoD's unexpected declaration of interest, last December. ESA hopes for an agreement among its members in April, in time for some industrial contracts to be let by midsummer.

Although the Columbus project, a manoeuvrable vehicle based on the space station, may appear to be a separate project, it will require a dedicated module integral with the space station as a whole.

European diffidence about DoD's possible involvement in the project centres on the extent to which scientific observations by Columbus and its crews may be restricted by US military requirements and on the unwillingness of some European governments to be seen to be contributing to a US-inspired military project in space.

Joseph Palca & Bill Johnstone

Embarrassing failure for Soviet launches

London

DURING the past few weeks, the Soviet Union has had problems with two of its Kosmos satellites. According to the Kettering monitoring group, K-1813, launched on 13 January, was destroyed after a failure of the rocket motor, and K-1817, launched on 30 January, failed to boost into a stable orbit and re-entered prematurely. In spite of the current policy of *glasnost* (openness), the Soviet space planners have been very reticent about the two Kosmos failures.

They have reason for reticence. Since the Challenger disaster last year, the Soviet Union has offered the services of its Proton launcher to countries wishing to launch communication, meteorological or experimental satellites on a commercial basis. The proposal has the special backing both of Mr Mikhail Gorbachev, who sees it as a first step to his plans for the 'star peace' based on international research projects which he expounded in India last year, and by Prime Minister Nikolai Ryzhkov, who in an interview with the TASS agency last month stressed the "versatile and reliable" nature of the Proton. In fact,



Soviet astronauts — still smiling.

up to the end of last year, according to official Soviet data, there had been seven failures of Proton out of 102 launches, and (in the absence of an official statement) it is impossible to pinpoint the cause of the two recent Kosmos failures.

Even without the commercial launches, however, Soviet cooperation programmes are booming. French and Syrian cosmonauts have been in training for some time, and the Bulgarians have at last been granted a re-run (scheduled in 1988) of their April 1979 mission, which was aborted when the Soyuz transporter failed to link up with Salyut station. Unmanned missions with international cooperation include a study of the surface of Phobos, X-ray astronomy, and research into solar activity. One difficulty for western scientists wishing to supply state-of-the-art equipment to joint missions (which could possibly run foul of the CoCom embargo on the export of sensitive technology to the Socialist Bloc) seems to have been partially overcome. In the case of commercial launches, the Soviet side has unequivocally promised that sealed experimental packages would remain sealed, and that there would be no attempt to "pump out technological secrets" from them. The same assurances could surely be extended to joint experimental missions. Vera Rich

Wellcome gears up production of AIDS drug

London

CONFIDENT that the first approval for the use of its anti-AIDS drug, Retrovir (trade name for AZT), is just around the corner, Wellcome plc is busily preparing itself for the anticipated demand. At a capital cost of £17 million, additional general purpose plants are being constructed both at Dartford, Kent, and at Greenville, Tennessee.

But even with their new production capacity, the company may not be able to meet estimated worldwide demand, in which case, presumably to avoid the kind of criticism that followed its initial allocation of the drug solely to the United States, Wellcome would allocate supplies on a geographical basis according to the number of reported cases.

Wellcome makes the drug (3'-azido-3'-deoxythymidine; AZT), by chemical synthesis from thymidine, which in turn is chemically synthesized by another company, whose identity is secret. Both companies are scaling up their chemical syntheses, which involve sixteen steps.

By May this year, Wellcome expects to have enough of the drug to meet the demand for its use in AIDS (acquired immune deficiency syndrome) patients who are seriously ill with pneumonia caused by *Pneumocystis carinii*, a group that has responded well to the drug in clinical trials. But if worldwide approval for use of the drug in patients with serious AIDS-related complex (ARC) was also soon granted, supplies would not meet demand. On existing figures there are about 15–20,000 patients in each category, needing an average of about one gram a day of the drug continually.

Approval for use of the drug in one or both conditions is expected from the US Food and Drug Administration in "five or six weeks", according to a Wellcome spokesman. And the UK Committee on Safety and Drugs is considering an application based on the same data. Wellcome will be submitting similar applications to other major European countries soon. In addition, clinical trials will test the value of the drug, alone or in combination therapy, in less severe cases of AIDS or ARC.

Responsibility for Retrovir production changed hands last week with the resignation of Dr Ronald Cresswell, who became the director responsible for coordinating Wellcome's US and UK research and development after the departure of Sir John Vane. No direct replacement has been made. Instead, Dr Trevor Jones takes over UK responsibilities and Dr Howard Schaeffer runs the US side.

Peter Newmark

US Navy torpedoes plan with NASA for ocean sensing satellite

Washington

THE US Navy is to scrap NROSS, the Navy Remote Ocean Sensing Satellite. The decision has not only dealt a telling blow to the future of the Navy's global weather and oceanographic forecasting, but has caused consternation among the Navy's partner agencies. The decision has forced NASA (National Aeronautics and Space Administration) to look for a new vehicle to carry a scatterometer, considered essential to the future of civilian oceanographic research.

NROSS began life in the 1985 fiscal year when it received formal budgetary approval. The satellite was to travel in a Sun-synchronous polar orbit, carrying a low-frequency microwave radiometer to provide all-weather high resolution measurements of sea surface temperature as well as an altimeter to measure waves and eddies. The Navy planned to include its own scatterometer on NROSS, but NASA offered to provide the Navy with NSCAT, an improved version of the scatterometer flown on the successful 1987 Seasat mission.

The Navy accepted and signed an agreement with NASA in July 1985. NSCAT transmits 14-GHz pulses towards the Earth, recording the reflected microwave signals. Ground-based computers interpret satellite telemetry to construct a map of ocean winds. NSCAT was to provide scientific data for NASA and operational data for the Navy.

The Navy's plans have been scuppered by rising costs in 1986. By July last year, the estimated cost of the satellite was \$420 million, \$150 million more than expected. By November, Assistant Secretary of the Navy Melvyn Paisley had called for a study of ways out of the dilemma, and the NROSS programme office presented three options at a meeting on 11 December: find the money from somewhere else in the Navy's budget, replace the low-frequency microwave radiometer with one costing less or cancel the programme.

Because the alternative radiometer would not work under cloudy conditions and with the prospects of finding the extra money only slim, it was decided to cancel the programme.

The oceanography "union" in the US Navy does not have the clout of others, such as those advocating construction of aircraft carriers or destroyers. There are also indications that the large cost increase for NROSS should not have come as a surprise, as the Navy had modified its requirements for the satellite. Thus, responding to concern created by the shuttle accident, the Navy had attempted to make contractors liable for mission failures, to

which contractors had responded by raising costs.

Attempts by supporters of NROSS to intercede have so far failed. Both NASA administrator James Fletcher and National Science Foundation director Erich Bloch wrote to Navy Secretary John Lehman urging him to retain the programme. Carl Wunsch, James O'Brien and Walter Munk, three of the four holders of Secretary of the Navy Research Professorships, also protested. Unpersuaded, Lehman agreed on 15 December to scuttle the satellite.

Following the Navy decision, Fletcher wrote to William Taft IV, deputy secretary of defence on 17 December, saying that "the cancellation of NROSS would set a bad precedent for other ongoing and future cooperative programs between DOD [Department of Defense] and NASA".

In a conciliatory response dated 20 January, Taft declined to change the decision but suggested that NSCAT might find a home in the Defense Meteorological Satellite Program, an option already being explored by NASA. The Air Force has now begun a study of that proposal, as well as the possibility of including NSCAT in DOD's Space Test Program. These options could be acceptable to NASA, since they would permit NSCAT to fly concurrently with TOPEX/POSEIDON, the joint NASA–French oceanography satellite due to be launched in December 1991. NASA is also investigating putting NSCAT on TOPEX.

Cancelling NSCAT, estimated to cost \$130 million, would be expensive for NASA. The agency has already spent \$30 million on the project, and an additional \$30 million would be needed to end the programme in an orderly fashion. But William Townsend of NASA's Oceanic Processes Program says he is "extremely optimistic" that something can be worked out to allow NSCAT to complete its mission.

Wunsch calls the Navy decision on NROSS "extremely short-sighted". He argues that cancelling NROSS will have severe repercussions for the Navy in the next 10–15 years when forecasting global oceanographic conditions will be critically important for new weapon systems.

D. James Baker, president of the Joint Oceanographic Institutions, says wind data provided by NSCAT are essential for WOCE, the World Ocean Circulation Experiment. Baker says a delegation of oceanographers is hoping to meet Navy officials soon to discuss the future of Navy's oceanographic programme.

Joseph Palca

Canada demands action from United States on acid rain

Washington

THE Canadian government's quarrel with Washington over acid rain has moved into a new and more acrimonious phase, with the Canadians demanding immediate action. At a meeting on 21 January, the Canadian Prime Minister, Brian Mulroney, gave US vice-president George Bush what is described as an "earful" and declared that the issue of acid rain will be "at the top of the agenda" for his meeting with President Ronald Reagan in April.

US efforts so far to reduce acid rain



were criticized in a report issued last month by Canada's environment ministry. The US State Department has responded with another high-level review while efforts to improve control continue to focus on the long-range development of new and affordable technologies to reduce the pollution from coal smoke, with no immediate reduction of the amount of sulphur dioxide crossing the border.

US policy on acid rain continues to ignore the Canadian demand that it should control the pollutants responsible for acid rain, despite the report in January 1986, by two specially appointed envoys, which persuaded the Reagan administration to agree in principle that acid rain was a serious problem. Since then, Canada has been waiting for policy changes in the United States, but the new budget contains no new programmes to reduce sulphur dioxide emissions from coal-fired plants.

Instead, the US administration points to its recent clean-coal technology legislation, which is intended to reduce the cost of new techniques for removing pollutants from coal smoke. But no immediate reductions are required, while the new technology will probably be applied only as older power plants need refurbishment.

The Canadians are not alone in calling for changes in US policy. Two bills introduced in the Senate this month would require a significant tightening of US air pollution controls. The bills were backed by testimony from physicians such as Dr Richard Narkewicz, president-elect of the American Academy of Pediatrics, who stated that "the ingredients of acid rain adversely affect the respiratory tract and consequently the quality of health for children" and "contribute greatly to mortality

rates". Further testimony indicated that air pollutants such as sulphur dioxide, nitrogen oxides and ozone put many others at risk, including people over 65 and sufferers from asthma and heart disease.

New political alignments in the Senate may prove to be insurmountable obstacles to the proposed legislation. Senate leaders from coal-producing states, for example, will probably provide strong opposition.

Although the administration no longer disputes the causal connection between sulphur dioxide pollution and environmental damage, it asserts that it is unclear at what rate the damage is being caused. "The argument can be made", said Chris Rice of the Environmental Protection Agency (EPA), "that the damage we see now is due to sulphur dioxide emitted in the 1950s or 1960s". But Debbie Sheiman of the Natural Resources Defense Council says "the administration seems to be using delaying tactics" to avoid using pollution

control technology now available.

Renewed Canadian demands for US reductions of acid rain have directed attention to Canada's own acid rain reduction programme. The seven provinces east of Saskatchewan agreed in 1985 to reduce sulphur dioxide emissions by 50 per cent by 1994. Stricter emission controls on automobiles will take effect in September this year.

But some in the US Congress believe the Canadians are being hypocritical, as they have yet to begin installing pollution control devices on their own smokestacks. A Canadian government spokesman rejects this charge as irrelevant, because most sulphur dioxide pollution in Canada results from non-ferrous smelters, not power plants. The most effective (and most often used) control device for such a smelter is to shut it down, as was done with the International Nickel Company's plant at Sudbury, Ontario, for example.

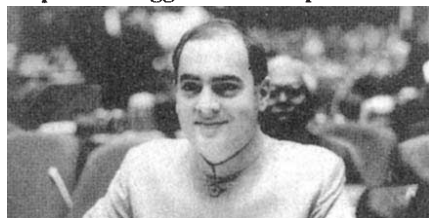
The question of what will happen to sulphur dioxide emissions in Canada when economic conditions favour the continued operation of these plants remains open.

Steven Dickman

Atomic power struggle under way in India

New Delhi

THE appointment of a successor to Dr Raja Ramanna, retiring chairman of the Indian Atomic Energy Commission (AEC), has been postponed at the last minute in the wake of threats of resignation and an internal power struggle between top scientists at



Mr Rajiv Gandhi

the Department of Atomic Energy (DAE). Whether an engineer or a physicist should be made head of the commission is the subject of a controversy that has brought to the fore the factional politics within DAE and the schism between research scientists and engineers. With two contenders vying for the post, an embarrassed Prime Minister, Mr Rajiv Gandhi, who is also in charge of atomic energy, has asked Ramanna to continue as chairman for a further month.

One of the aspirants for the post is Dr P.K. Iyengar, the director of the Bhabha Atomic Research Centre (BARC), which is the principal agency responsible for research and development. Iyengar, a reactor physicist and a key figure in the design and testing of India's first nuclear device, was Ramanna's student and his favourite for the post. The other contender is Dr M.R. Srinivasan, an engineer, who is in charge of DAE's nuclear power board,

which is responsible for the design, construction and operation of nuclear power plants. Both Iyengar and Srinivasan are members of AEC.

Ramanna, who was to have retired on 31 January, was not told of his successor until the previous evening when he was told to hand over to Srinivasan. Twelve hours later the order was withdrawn, apparently because Iyengar threatened to resign. Ramanna was then asked to continue as chairman until the end of February and the prime minister presumably hopes that the delay will give him time to pacify Iyengar. Scientists at DAE, upset over the last-minute change, have openly accused Ramanna of blackmailing the government and stalling the appointment of Srinivasan, whose name had been proposed by a special selection committee.

Gandhi's handling of the issue has been attacked in the newspapers, which claim that it has lowered his credibility within the scientific community. Similar criticisms were voiced a few months ago when Gandhi abruptly removed Dr S. Varadarajan from his post as director-general of the Council of Scientific and Industrial Research.

The bitter taste left behind by the struggle over the succession at AEC will take time to wipe out, but Gandhi has already taken steps to bring peace among the warring scientists. He has held separate meetings with Iyengar, Srinivasan and Ramanna, and the name of the new AEC chairman is expected to be announced soon.

K.S. Jayaraman

Ten colleges combine forces to strengthen Earth Sciences

Washington

TEN small liberal arts colleges which have already had a disproportionate impact on graduate research in the Earth sciences are hoping to improve that record with a \$450,000 grant from the W.M. Keck Foundation. The grant to the ten-college consortium will permit joint faculty-student research projects as well as short-term faculty exchanges.

Four-year liberal arts colleges have regularly provided a large number of graduate students to US universities (see *Nature* **322**, 203; 1986). This is borne out by a 1982 survey of 876 undergraduate institutions conducted by Franklin and Marshall College, a member of the ten-college consortium. The survey showed that although the ten colleges now banding together produced less than one per cent of the total number of first degrees in the institutions surveyed, they accounted for more than a quarter of the total graduate degrees in the Earth sciences.

The colleges in the consortium are Amherst (Massachusetts), Beloit (Wisconsin), Carleton (Minnesota), Colorado College, Franklin and Marshall (Pennsylvania), Pomona (California), Smith (Massachusetts), Whitman (Washington), Williams (Massachusetts) and College of Wooster (Ohio).

Reinhard Wobus, a geology professor at Williams College in Williamstown, says

the strength of the small colleges comes from the opportunities for close faculty-student interactions. The Keck grant will offer top undergraduates in the Earth sciences a chance to participate in professional research. In the spring following each summer's research projects, there will be a conference to discuss the findings.

Wobus says a similar programme sponsored by the National Science Foundation in the early 1970s involving Williams and three neighbouring colleges proved the value of such experience. If there is a problem with the new programme, Wobus believes it will be logistical. The ten colleges are scattered throughout the United States.

Improving instructional skills is also a goal of the consortium. Faculty from the colleges will go on three-day visits to the other institutions to observe classes and conduct guest lectures.

The first research projects will begin this summer. One, directed by Wobus, will collect and analyse material thrown up by volcanoes in the Thirtynine Mile volcanic field in central Colorado. Other projects this summer will be a study of ancient stream systems in Montana and carbonate sedimentation on San Salvador island in the Bahamas. Although the programme will be limited to three research projects this summer, the number should double in 1988.

Joseph Palca

Japan sets world record for levitation

Tokyo

JAPANESE National Railways (JNR) established a world record last week when the magnetically levitated (Maglev) train reached a speed of 400 km an hour with a crew of three abroad. The successful

experiment sets the stage for test runs of a prototype commercial vehicle later this year.

The new record was made possible by doubling the electrical capacity of JNR's 7-km test track at Miyazaki, Kyushu, to 6,000 V, and shattered the previous record of 350 km per hour for a manned train set by West Germany's Transrapid 06 vehicle in December 1985.

Japan's MLU-001 experimental train 'floats' on on-board superconducting magnets and is propelled forward by magnetic coils set in the sides of the trackway. A prototype commercial vehicle capable of carrying 44 passengers at a speed of up to 420 km an hour will begin tests in March on the Miyazaki test track, and Yoshihiro Kyotani, manger of the Maglev project, hopes that a new 50-km trackway for the prototype will be constructed by the end of this decade. Seven prefectures, including Hokkaido, Yamanashi and Saitama, have already expressed interest in the new line which will cost at least ¥150,000 million (\$1,000 million) to build and can be put into commercial use once experiments are completed.

David Swinbanks

Japan under fire from US on supercomputers

Washington

SUPERCOMPUTERS are the latest Japanese products to come under scrutiny by the Office of the US Trade Representative, this time at the behest of the White House. The results of an investigation begun last year will be disclosed next month.

The President's Trade Strike Force, an informal body at cabinet level, asked for the Trade Representative's investigation, apparently in response to the first purchase of a Japanese supercomputer by a US corporation. The Houston Area Research Center (HARC) — a non-profit corporation designed to link government and industry with the research capabilities of local universities — announced last year that it was acquiring a Nippon Electronics Corporation (NEC) SX2 supercomputer for \$20 million. The prospect of Japan successfully invading another market hitherto dominated by indigenous companies evidently provoked the request for an investigation.

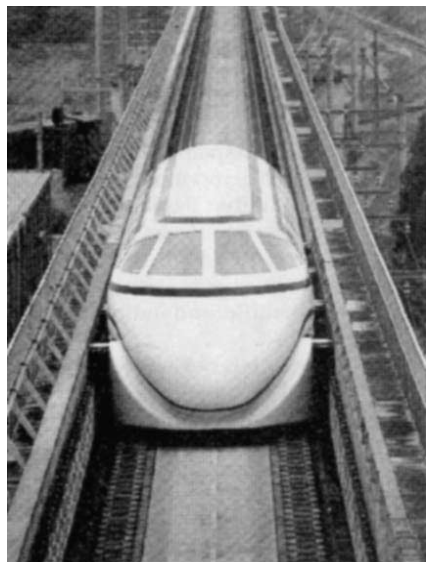
The investigation is being carried out in parallel with the Market Oriented Sector Selective (MOSS) talks with Japan, which deal with the Japanese markets from which the United States has been excluded. These include pharmaceuticals, electronics, lumber, automobile parts and telecommunications equipment. Supercomputers have now been added to the electronics segment of the talks which apparently are going 'slowly'.

Two US companies now manufacture supercomputers; Cray Research Inc. and Control Data Corporation (CDC). Cray has sold six supercomputers in Japan, the latest of them last month, when Honda bought a \$5.5 million Cray X-MP/1. The X-MP/1 has only a quarter of the power of its big cousin, the X-MP/4, but is still 50 per cent more powerful than the Cray 1. So far, CDC has not sold any supercomputers in Japan.

Bob Gaertner, vice president for communications for Cray Research, says Cray's problems in Japan have been chiefly in sales to government agencies and universities. Cray's Japanese sales total \$60 million, all to the private sector. Gaertner says Cray wants to expand Japanese sales and is "anxious to have a more open market". So far, Cray has sold 24 of its top-of-the-line supercomputers worldwide; 18 X-MP/4s and six Cray 2s.

HARC's SX2 can perform 1,300 million floating point operations per second (1.3 gigaflops). The new CDC ETA/10 operates at only 10 gigaflops, and costs between \$15 and \$21 million depending on how it is supplied.

Joseph Palca



The new high-speed Maglev train.

Britain's doctors in legal wrangle over confidentiality of records

London

BRITAIN'S medical profession is embroiled in the debate over the ethics and legality of withholding information from patients. Tests to determine the health of fetuses and others to identify AIDS (acquired immune deficiency syndrome) carriers has highlighted deficiencies in British medical law.

The fetal test debate was originally provoked by a report from India of concern over the "steady increase in the incidence of female feticide", brought about by abortions inspired by information derived from amniocentesis testing (*Nature* 324, 202; 1986). The controversy continued with correspondence, in response to the Indian report (*Nature* 325, 190; 1987), from British cytogeneticists who have been withholding sex information culled from amniocentesis.

The debate has been reopened by the council of the Association of Clinical Cytogeneticists, which has become increasingly concerned over the potential misuse of amniocentesis data. Some members of the association have advocated withholding fetal sex details from parents who may demand abortion if the fetus is of the 'wrong' sex.

Last week, the council sought to determine the scale of the problem. Such evidence as could be acquired was 'anecdotal' and the association has been unable to issue definite guidelines. The full legal implications of withholding information from patients are to be investigated; in the meantime, the guidelines will be those agreed between "each regional laboratory and regional clinical colleagues".

While the UK Abortion Act theoretically does not allow the abortion of a fetus

on the grounds of unwanted sex, health authorities have become increasingly concerned that the problems of India may be repeated in Britain, particularly among certain ethnic groups. Because of the scale of the problem in India—in a recent study of 8,000 abortions, 7,997 fetuses were female—new legislation is being drafted to restrict the practice of amniocentesis and other methods of determining fetal sex.

The screening of all hospital patients for the AIDS virus has raised similar legal and ethical questions. The UK government is considering authorizing the 'blind testing' of all patients to identify the AIDS virus. No individual may be identified in the tests and the information will be used only to establish the spread of infection. The disclosure was made last week by the government's chief medical officer, Sir Donald Acheson, in evidence to the House of Commons Social Services Select Committee.

He said: "It is being considered by ministers as a possibility. It would help us very much in planning and getting an idea of the prevalence and trends of the infection."

The plan has provoked much controversy. The British Medical Association supports the plan but the Medical Research Council, which this week will give evidence to the Commons committee, has raised doubts about the legality of such a strategy.

Bill Johnstone

• Britain has increased its annual budget for treating AIDS from £3 million to £7 million; the major regional health authorities treating AIDS believe a figure of £16 million would be a more realistic estimate of what is needed. □

Peace attempt with university pay deal

London

BRITAIN'S university lecturers have been offered a two-year pay deal that will raise the average salary immediately by 16 per cent, backdated to last December, and by a further 7 per cent in March 1988. The figures are based on existing salary rates.

The executive is to meet this week to endorse or reject the offer although the negotiators have recommended its acceptance. About 47,000 academic staff and certain support staffs will be affected by the deal. The Association of University Teachers which had claimed 6 per cent, to be effective from last April, and 18 per cent from April this year, considers it the best deal under the circumstances.

According to Ms Diana Warwick, General Secretary of the AUT: "this offer is no

remedy to recruitment and retention problems within our universities; it won't staunch the brain drain. There's been no pay increase in universities since 1985. It's clear that we have wrung out of the vice-chancellors the best deal we could get, within the miserly terms allowed for by the Secretary of State."

The monies will come from the £71 million of government funds allocated to the universities in a three-year plan—£40 million in 1987–88, £16 million in 1988–89 and the remainder in 1989–90.

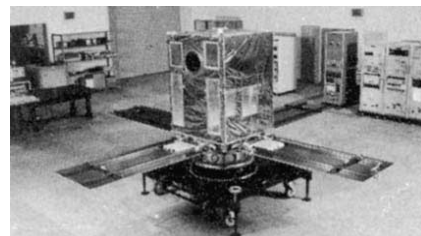
The AUT will open negotiations on a new claim in April 1988, just one month after the award of the second tranche. The union insist that the deal is a two-year agreement and must not be construed as running after March 1988.

Bill Johnstone

Japanese X-ray satellite goes up successfully

Tokyo

JAPAN'S X-ray astronomy satellite, Astro-C, was successfully launched from the Kagoshima Space Center of the Institute of Space and Astronautical Science (ISAS) on 5 February. Developed jointly by Japanese, British and US scientists, the



Astro-C

probe will monitor faint X-ray sources in the far reaches of the Universe.

The satellite, named Ginga (galaxy) in orbit, was carried aloft by a 28-m M-3SII solid-fuel rocket at 3.30 p.m. About 6 minutes later, the 420-kg satellite entered low Earth orbit, and radio transmissions from the probe were picked up after it had completed its first loop around the Earth at an altitude of 500–600 km.

Ginga, ISAS's third X-ray satellite, is the world's only operational X-ray probe following the demise of the European Space Agency's EXOSAT early last year. It carries eight identical large-area proportional counters, developed with groups at the University of Leicester and the Rutherford Appleton Laboratory in the United Kingdom, an all-sky X-ray monitor, and a gamma-ray burst detector, made in collaboration with a group at Los Alamos National Laboratory in the United States.

Collaboration between ISAS and the Leicester group stretches back seven years. Scientists have been exchanged under UK/Japan schemes for visiting scholars; the British side developed the hardware, Japan the electronics. Similar arrangements were made with the United States. The total cost of Ginga, including the MU-3SII rocket, is estimated at ¥7,300 million (about \$49 million).

The highly sensitive, large-area detectors (4,500 cm² in total) will be trained on distant X-ray sources, in particular quasars. The time-scale of X-ray fluctuations will give an indication of the size of the quasar nucleus and, according to Minoru Oda, director of ISAS, strong fluctuations would provide good evidence for the existence of a black hole in the centre of quasars that sucks in stars and interstellar material, releasing vast amounts of energy in the form of X-rays.

David Swinbanks

International spaceports planned for northern Australia

Sydney

AUSTRALIA is to be the site for an international spaceport, if the governments in the top end of the continent have their way. A A\$93,000 study commissioned by the Queensland state government is to recommend that at least 2,000 km² be set aside for a spaceport on the Cape York Peninsula, Australia's northernmost tip.

The size of the area is to allow plenty of space for horizontal take-off and landing rocketships, such as the British HOTOL or West German Sanger projects. Cape York is a vast and virtually unpopulated area near to the equator, at a latitude of 12°S and is close enough for rockets launched there to take full advantage of the boost provided by the Earth's spin.

The same applies to the north coast of the Northern Territory. Having been prompted to consider the benefits of a spaceport, the Northern Territory government has dusted off a 1965 report by the European Launcher Development Organization (ELDO) which recommended a launch site near Darwin. A committee is reviewing the conclusions of the ELDO study to see if they are still valid today.

Both governments hope to attract launch operators from around the world. They consider that US rocket manufacturers will be moving into the launch business now that NASA is no longer launching commercial satellites.

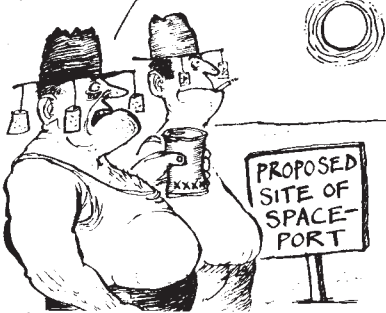
The west Pacific location should be particularly attractive to Japan whose only launch sites are 30 degrees off the equator and closed for 10 months a year to placate local fishermen. They are also looking hopefully towards China whose only launch site at present is 40 degrees from the equator. The Queensland study is being conducted for Australia's Institution of Engineers by representatives of Hawker De Havilland, British Aerospace Australia, the University of Queensland, AUSSAT (the company operating Australia's domestic communication satellites), and the Commonwealth Scientific and Industrial Organization.

According to Mr Stan Schaetzel, leader of the study group and technical director at Hawker De Havilland, the report will recommend a site on the western side of Cape York facing the Gulf of Carpentaria. One great advantage of this site is its access to a variety of orbits without needing to overfly any centres of population. A standard due east launch would carry the rocket over the uninhabited forests of Cape York then out over the Pacific. For a polar orbit the rocket would head SSW, flying down over the Gulf of Carpentaria, across the continent and out over the Southern Ocean on a heading for the

South Pole. Spent first-stage boosters would crash into the Simpson Desert somewhere to the east of Alice Springs. A course across Cape York and closer to the Queensland coast gives the spaceport access to a 28.5° orbit which corresponds to a due-east launch from Cape Kennedy and is the orbit NASA's space station will occupy. The US spaceports have very poor access to some orbits. Polar orbits cannot be reached from Cape Kennedy without wasting a lot of energy, whereas the other US launch facility at Vandenberg Airforce Base in California can launch into orbits but not due-east. Schaetzel points out that the US space agenda announced in 1986 calls for the establishment of a Moon base by 2010. The 28.5° orbit may well grow in importance in terms of the space station and space-based manufacture and for missions to the Moon and beyond.

Cape York's climate is favourable; only 39 launch days would be lost to electrical storms each year compared to 57 days lost

I'd still rather have the Americas Cup...



at Cape Kennedy and 89 days at the site run by the European Space Agency (ESA) at Kourou in French Guyana, at present the only equatorial launch site.

Provision of infrastructure in such a remote part of Australia is simplified by the fact that the prospective spaceport is right alongside what are perhaps the world's largest bauxite deposits. A nearby port capable of handling ships of up to 75,000 tonnes was built by Comalco Aluminium to support the workers at its mine there. Australia can also offer good radar cover and air traffic control as well as guaranteed political stability.

The world can be thought of as being divided into a land hemisphere and a water hemisphere. At present, all the world's launch sites are in the land hemisphere. Australia is in the water hemisphere. According to Schaetzel, at very least Australia an expect to play host to a spaceport which provides the pilots of future generations of shuttles with an emergency landing site.

Charles Morgan

Far Eastern Science Centre performs badly

London

THE Far Eastern Science Centre of the Soviet Academy of Sciences, founded in 1970 as a focus for scientific and technical progress on the Soviet Pacific Coast is not living up to expectations, according to *Pravda*. Academician Skryabin, the academic secretary of the Soviet Academy of Sciences, blames the "sickness" of the centre on the lack of adequate "cadres" but locals (and *Pravda*'s Vladivostok correspondent, N. Bratchikov) say the distance from the academy's headquarters in Moscow makes proper control difficult.

New institutes of the Far Eastern Science Centre are built extremely slowly, and when completed, frequently stand unoccupied for lack of equipment. Only about 30–50 per cent of resources allocated are actually applied to work for the centre. The situation, bad in Vladivostok, is even worse in the outlying establishments of the centre. Laboratories, libraries and a computer centre specified in the plans have not been built. Furthermore, although some 9,500 staff (including 2,400 scientists) are now employed the number should be twice that. But the centre cannot attract qualified and motivated personnel until proper services are provided. At present 2,500 employees of the Far Eastern Science Centre are waiting for apartments and there are no hospitals, kindergartens or leisure facilities. The institutes do not solve such problems, nor do they provide proper working conditions. Laboratory space is only two-thirds of that given to a scientist at the Siberian Branch of the Academy in Novosibirsk, and allocations for instruments and equipment are only about half those at Novosibirsk.

In the mid-1970s, the centre was cited as an example of slackness in outlying scientific establishments. Mr Mikhail Gorbachev's recent visit to the Far East, where he repeated his calls for "restructuring", and "openness" has focused attention once again on the defects of the centre. However, the centre can claim a sound research record in many fields, especially Earth sciences, oceanology and ichthyology; its Kamchatka vulcanology centre is a world leader.

But the scientists say that if they are to do a proper job the centre should be converted into a Far Eastern branch of the academy (analogous to the Siberian branch) with an autonomous presidium, it should have a "worthy" material and technical base, and, while the planned new institutes should be built, the existing institutes should be properly equipped and staffed.

Vera Rich

Japanese elders lose their grip on university appointments

Tokyo

JAPAN'S Science and Technology Agency has introduced a radically new research system that breaks with the tradition of tenure for academics.

The International Frontier Research System was set up last year at the Science and Technology Agency's Institute of Physical and Chemical Research (RIKEN) near Tokyo with funding of ¥1,100 million (\$7 million) in the first year rising to ¥1,500 million in fiscal year 1987. The programme, scheduled to run for 15 years, consists of 40 researchers in seven teams, split into two groups, "Frontier Materials" and the "Biological Background of Homeostasis". A group to study the artificial brain may be added later.

The rigid system of tenured appointments has long been regarded as a weakness of Japan's universities and research institutes. Professors, once established in a chair, remain entrenched until retirement and enjoy almost dictatorial power over younger researchers who wait patiently in line for a post to be vacated.

Attempts have been made to introduce greater fluidity and flexibility into the system. When the Okazaki institutes for joint university use were set up there was heated debate over whether professorial appointments should be non-tenured. But in the end the only change was a system of adjunct professorships.

The Frontiers system parts from tradition in two respects. About one-third of the researchers, including three of the team leaders, will be non-Japanese. Second, all researchers will be employed under one-year contracts renewable up to a maximum of five years.

The system aims at funding basic research by young scientists that will "sow the seeds of technology in the 21st century", according to Takashi Bito, deputy director of the programme. Patent rights for technology arising out of the research will be shared equally between the Japanese government and the inventor. Frontiers thus in some respects resembles the agency's ERATO programme (see *Nature* 305, 373; 1983); although Bito says ERA-TO is more technology-oriented.

The homeostasis group has four teams. Gabriel Gachelin of the Pasteur Institute of France will head the chromosome team, which will study homeostasis and ageing at the gene level. Koichi Chitani, formerly of the University of Washington, heads the ageing process team, which will concentrate its research efforts in the first year on proteins associated with Alzheimer's disease, such as paired helical filaments. Masaki Furuya will resign from Tokyo University to lead research by the

plant homeostasis team into phytochromes, and Tomotari Mitsuoka's intestinal flora team at RIKEN will continue to investigate the role of intestinal bacteria in health, nutrition and ageing.

With the rapid 'greying' of Japanese society (see *Nature* 321, 553; 1986), politicians, academics and pharmaceutical and biotechnology companies are turning their attention to the problems of old age. Researchers from Snow Brand Milk Products, Meiji Milk Products and Nissin Flour Milling Co. will join the intestinal flora team, and special facilities will be set up at RIKEN for raising aged mice and rats.

The three teams of the Frontier Materials group will carry out basic research into the development of new electronic and optical devices. RIKEN's Susuma Namba will head the quantized elements and devices team which will use electron, laser, molecular and ion beams and atomic-layer epitaxy to develop ever smaller electronic devices (such as integrated circuits and Josephson Junctions). Already down to the 100 Angstrom level, Namba works in the realm where quantum effects and electron-wave interference can be observed, and he foresees a new line of engineering emerging from his research, based on electron waves as opposed to electromagnetic radiation.

The non-linear optics and advanced material research team will be headed by Anthony Garito of Pennsylvania University, and will aim at developing non-linear optical devices and two-dimensional superconductors using organic materials, such as methyl nitro aniline. Kevin Ulmer of the Center for Advanced Research for Biotechnology at Maryland State University will lead research by the bioelectronic materials team into electro- and photo-active proteins from which should emerge new biosensors and, perhaps, the biochip. Canon Inc., Ajinomoto Co., Unitika Ltd and other Japanese and US companies are interested in joining the two teams.

But although there seems to be no lack of researchers for Frontier Materials, some of the Homeostasis teams concerned with basic biology are finding it hard to recruit members. Young and talented Japanese researchers, although initially interested, balked at the prospects of having to find a new job after five years. Problems of job security do not arise for the Japanese team leaders themselves — all are at or approaching retirement age, and several hold parallel appointments outside the Frontiers system. A true revolution will have occurred only if younger leaders are appointed when the system undergoes a turnover in staff five years hence.

David Swinbanks

4,000 Chernobyl deaths?

A NEW report from US government estimates that there will be 4,000 excess cancer deaths in Europe and 10,000 in the Soviet Union as a result of the Chernobyl disaster. The report was prepared by the Nuclear Regulatory Commission in the United States. The excess cancers will probably be indistinguishable from the 70 million cancer deaths that are predicted in the European area affected by the fallout over the next 70 years. Chernobyl-induced cancers might be more noticeable in the Soviet Union, where 9.5 million deaths from cancer are otherwise predicted. Estimates of the number of deaths have varied widely; these figures lie at the conservative end of the spectrum. □

Soviet drilling project snag

A US DELEGATION poised to travel to Moscow last weekend to sign a memorandum of understanding making the Soviet Union an official member of the Ocean Drilling Program was ordered not to leave just one day before the scheduled trip. The Defense Department postponed the signing by raising concern about technology transfer through Soviet participation in the project. The Soviet Union would have been the eighth participant in the project, and would have contributed more than \$1 million in membership dues for the current fiscal year. □

Cold closes nuclear post

ONE of the three 'nuclear-monitoring' stations installed by a US team in Kazakhstan last year has been put out of action as a result of the severe cold last month. On the night of 16–17 January, the low temperatures at the Bayan-Aul station produced a short circuit which started a fire. Although the monitoring instruments installed in the boreholes remain unharmed, the fire destroyed a building containing all the recording equipment, without which the monitoring programme cannot function. The staff quarters at the site were also destroyed, without loss of life. □

Solar power for Cuba

Dr Rosa Elena Simeon, president of the Cuban Academy of sciences, has formally inaugurated Cuba's prototype solar power plant at the Santiago de Cuba solar energy research station.

Cuba has no fossil fuels and its rivers are insufficient for normal hydroelectric generation. Accordingly, as President Fidel Castro stated last July, the construction of thermal power stations will stop, to be replaced by nuclear power, boosted by pumped storage hydroelectricity at peak periods, and some solar power. (Cuba's first nuclear power plant at Gienfugos will have four 4,000 MW Soviet VVER reactors, and will be 'complemented' by a pumped storage plant at Fomento.) □

Australian deserts defended

SIR—Kenneth Mellanby has recently expressed his views on conservation issues in Australia (*Nature* 325, 112; 1987). By appearing in *Nature*, these views might be given greater credence than they perhaps deserve; although Mellanby is entitled to his opinions, certain statements in his article cannot be left unchallenged.

In late 1986, the federal government did nominate Stage 2 of Kakadu National Park for inscription on the World Heritage List. At the last moment, this nomination was temporarily withdrawn although not as a result of representations from the Northern Territory Government or its lobbyists but as a consequence of legal action taken in the federal court by a mining company.

Mellanby apparently finds much of the landscape of the Northern Territory either "boring" or "dull" — scarcely an objective assessment, nor one universally shared. No doubt many people do find deserts boring; nevertheless, they offer landscapes that others find of peculiar beauty while the biological diversity and the adaptations of the biota to the environment are of considerable interest to both pure and applied scientists. To suggest that eucalypt forests (although technically many of the eucalypt communities in the Northern Territory are woodland) are uniform and dull is to view them with remarkably unperceptive eyes. These ecosystems, which, with the exception of limited areas of eucalypt savanna in Timor and Papua New Guinea, are uniquely Australian and support a great diversity of organisms. A first fleeting glance might suggest a degree of uniformity but anyone who takes the time to look more closely cannot but be impressed by the variety and complexity of these communities. To have described Stage 2 of Kakadu as "the most boring national park in the world" is a greater reflection on the writer than on the area.

Natural properties submitted for inclusion on the World Heritage List are judged against four criteria, of which Professor Mellanby selectively quotes from one only. The full criteria say such properties must:

(i) be outstanding examples representing the major stages of the earth's evolutionary history. This category would include sites which represent the major 'eras' of geological history such as 'the age of reptiles' where the development of the planet's natural diversity can well be demonstrated and such as the 'ice age' where early man and his environment underwent major changes; or

(ii) be outstanding examples representing significant ongoing geological processes, biological evolution and man's interaction with his natural en-

vironment. As distinct from the periods of the Earth's development, this focuses upon ongoing processes in the development of communities of plants and animals, land-forms and marine and fresh water bodies. This category would include for example (a) as geological processes, glaciation and volcanism, (b) as biological evolution, examples of biomes such as tropical rainforests, deserts and tundra, (c) as interaction between man and his natural environment, terraced agricultural landscapes; or

(iii) contain unique, rare or superlative natural phenomena, formations or features or areas of exceptional natural beauty, such as superlative examples of the most important ecosystems to man, natural features (for instance, rivers, mountains, waterfalls), spectacles represented by great concentrations of animals, sweeping vistas covered by natural vegetation and exceptional combinations of natural and cultural elements; or

(iv) be habitats where populations of rare or endangered species of plants and animals still survive. This category would include those ecosystems in which concentrations of plants and animals of universal interest and significance are found.

(From Nomination form for inclusion of properties on the World Heritage List.)

It is interesting to note that under criterion (ii) "boring" deserts could be considered, while under criterion (iii) Stage 2 of Kakadu certainly offers "sweeping vistas covered by natural vegetation" even if much of it is "dull and uniform" eucalypt communities. I am not aware of the details of the federal government nomination of Kakadu Stage 2. Nomination in itself does not guarantee inscription on the list; nominations are subject to a detailed refereeing process as well as examination at the World Heritage Committee itself. When it comes to assessment of natural beauty, then decisions are bound to be subjective; although one would expect the committee to be impartial, its members would not necessarily show the same biases as Mellanby. In judging nominations against other criteria, more objective assessment is possible.

The nomination of Stage 2 of Kakadu has become part of a political agenda that goes far beyond concern for environmental matters. However, it is not possible to dismiss the case for nomination out of hand as being a mere political ploy.

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Rem overdose

SIR—The communication by R. Russell-Jones on "Cancer risk assessments in light of Chernobyl" (*Nature* 323, 585; 1986) continues the rapid calculations made following the disaster. It also is technically incorrect as well as misleading. Presumably the 400,000 rem (4,000 Sv) quoted are the product of the mean "dose" (more accurately "dose equivalent") and the number of people (that is, the collective dose equivalent in "man-rem"), and the UK population might over the next five years perhaps receive an average of 10 mrem. This would be a mere two per cent of the natural background dose.

Having been a member of the BEIR III Committee, I know very well that we stated that the effects of background radiation are unknown and that we refused to provide any risk estimates for doses that are less than 10 rem.

Although there are fairly firm scientific reasons for believing that there is proportionality between dose and genetic effects, this so-called 'linear hypothesis' lacks any substantial justification in the case of radiation carcinogenesis and seems in fact most dubious in view of the complexity of the process. It may possibly serve as a crude estimate of the risk attendant to maximum permissible radiation doses but extrapolations by many orders of magnitude, whether made by Russell-Jones or others, are scientifically indefensible.

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The calchemists

SIR—The increasing use of computers to simulate the properties of chemical entities has led to an unnecessary proliferation of new scientific terms that are unpleasant on the ear. For example, "drug design by computer-based molecular docking", or "molecular dynamics calculations of bulk properties of..." or "computer graphics simulation of solvent-protein interactions" or "computer-generated electrostatic surface representations", are far too latinized to use in normal conversation. I propose that we replace such uses by the historically more suitable term "calchemy" and its derivatives "calchemic", "calchemistry", "biocalchemistry", and begin to raise funds for the formation of new academic departments.

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Joint hunt for missing particles

Cosmologists and high-energy physicists, renowned for radical imaginativeness, seem to have infected solid-state physicists with enthusiasm for the search for dark matter.

ASTROPHYSICISTS and high-energy physicists alike are just now frustrated in their search for missing matter, the former because they have good reason to suppose matter to be present where they cannot see it, the latter because their descriptions require particles not yet found. The realization that both camps may after all be hunting the same entities has already proved stimulating. It is even more enticing that condensed matter physics, pushed to new limits, may be cheaply used to find the elusive stuff.

Dark matter of one kind or another has been perplexing astrophysicists and cosmologists for some time. Evidence for its existence is still circumstantial — the anomalous orbital behaviour of stars in galaxies and, on a grander scale, theoretical prejudice (otherwise called the inflationary model of the very early Universe) that the mean density of the observable Universe should be about one hundred times that so far detected with visible or other radiation. Of the two principal candidates — light relativistic ('hot') neutrinos and more massive ('cold') particles — the second seems to fit the constraints better.

High-energy physics has its own dilemma. What is called the 'standard model', a combination of quantum chromodynamics (QCD), with its quark-gluon description of strongly interacting particles, and the gauge theory of electromagnetic and weak interactions, has made many successful predictions and is not contradicted by observation. But there remain some 20 free parameters while the standard model requires the existence of new particles yet to be detected.

One such particle stems from what might be called a minor embarrassment of QCD. Unmodified, the theory would prefer the electric dipole moment of the neutron to be far greater than the tiny upper limit observed. To circumvent this difficulty, a new hidden symmetry has been imposed within the theory which, when broken at the appropriate energy, results in a massive particle called the axion.

This sounds arbitrary and aesthetically unsatisfying, but such shortcomings are outweighed by the explanatory success of QCD. Less experimentally compelling, but at least as appealing to their supporters (though, as the theorist John Ellis once remarked, mother aardvarks find their offspring appealing too), are the supersymmetry theories. Alas, their price for unifying three or all four of the funda-

mental interactions is a plethora of putative particles.

Here is but one aspect of the cross-fertilization of astronomy and high-energy physics: axions and supersymmetry particles have masses and other properties that make them particularly strong candidates for the cold dark matter, not only pervading the Universe as a whole but also gravitationally bound along with visible matter within galaxies. Galactic dark matter would be streaming through our laboratories but, to be detectable, would have to react sufficiently strongly with ordinary matter by means other than gravity.

Happily, it should. (See, for a review, P.F. Smith, Proceedings of the second CERN-ESO symposium, European Southern Observatory, 1986.) The axion, for example, should interact with a magnetic field to yield a photon. For the expected range of masses, an array of microwave cavities in a five-tesla field could be designed to detect the narrow signature of galactic axions somewhere in the very broad band between 1 and 1,000 GHz.

More widespread interest has been generated by the prospect of catching supersymmetry particles. Not only are the rewards potentially great, but the combination of disciplines involved is entertainingly unusual. In particular, solid state physicists are responding to the novel challenge of how to detect and identify the perturbation in a crystal lattice following the scattering off a nucleus of a dark matter particle. At low temperatures, the recoil of the nucleus generates quantized acoustic waves, or phonons, in the lattice. The trick is then either to detect the ensuing temperature rise or the phonons themselves.

The former is the approach adopted by Bernard Sadoulet and others at Berkeley and by Peter Smith and collaborators at the Rutherford Appleton Laboratory near Oxford. They take advantage of the exceedingly small heat capacity of tiny pure crystals at 10–20 mK: a single 6 keV photon can be detected by the 0.1 per cent temperature rise that it produces.

Different supersymmetry particles (sneutrinos, higgsinos and photinos seem to be the favourites) will give a variety of energy spectra according to the atomic mass and spin of the target nuclei, hence the planned simultaneous use of materials such as silicon, gallium arsenide and lithium bromide. Another signature is the seasonal variation in the particle spectrum

caused by the Earth's orbital motion relative to the galactic flux. Such experiments will have to be conducted underground to ensure adequate shielding from cosmic rays.

There are other, perhaps more imaginative and even elegant, ways of detecting a nuclear recoil, such as that developed by A. Drukier and others at the Max Planck Institute at Munich. He proposes to suspend granules of superconducting material within a magnetic field, setting the temperature so that a recoil within a granule would flip it into its resistive state, causing a detectable movement of magnetic flux.

The temperature rise in such small detector crystals (typically of micron thickness) arises from the multiple scattering of phonons off surfaces. Nobody knows for sure what happens if, as might be desirable, the crystal size is increased. Low-energy phonons, at least, experience little scattering within the crystal while the role of surface scattering would diminish, but nobody knows the energy spectrum of phonons generated by a recoil or how all the possible modes will propagate. Furthermore, the scattering properties of surfaces are very sensitive to the presence of small numbers of external atoms of contaminant.

With such uncertainties in mind, ambitious plans have been put forward to detect phonons directly. One being developed by Blas Cabrera and others at Stanford exploits the effect of phonons on a superconductor. If the phonons have sufficient energy they can disrupt the 'Cooper' pairs of electrons which, by means of their interactions with the lattice, otherwise flow freely. The disruption leaves a combination of holes and electrons which can in turn be detected by means of a tunnel junction.

What are the prospects? Even the furthest developed nuclear recoil detectors will take two to three years to become operational. Thereafter, supersymmetry particles may either be found immediately or, if the seasonal signal is required for convincing detection, only after a further year or two. But perhaps they will not be found at all. Agencies being asked to finance these shots in the dark would be right to take the gamble; gamblers elsewhere should bet on the last possibility, but if they are wrong, the excitement would surely compensate for the hole in their pocket.

Philip Campbell

Astronomy

Giant luminous arcs discovered in two clusters of galaxies

Bohdan Paczyński

ONE of the highlights of the 169th meeting of the American Astronomical Society, held in Pasadena, California on 4–8 January 1987, was the remarkable discovery¹ reported by R. Lynds (Kitt Peak Observatory) and V. Petrosian (Stanford University): “We announce the existence of a hitherto unknown type of spatially coherent extragalactic structure having, in the two most compelling known examples, the common properties: location in clusters of galaxies, narrow arc-like shape, enormous length, and situation of center of curvature toward both a cD galaxy and the apparent center of gravity of the cluster. The arcs are in excess of 100 kpc in length, have luminosities roughly comparable with those of giant E galaxies, and are distinctly bluer than E galaxies — especially so in one case”. The two clusters (see figure) are Abell 370, with a known redshift of 0.373 (ref.2) and 2242-02, for which no redshift has been published. The structures are different from anything yet seen, but nobody doubts that the objects are real.

Lynds and Petrosian found only two cases of the arcs in a sample of 57 clusters. J. Gunn (Princeton University Observatory, PUO) and D. Schneider (Institute for Advanced Study, Princeton, IAS) did not see anything like that in their sample of about 400 clusters of charge-coupled display images obtained on a 200-inch telescope. There are certainly many theoretical speculations about the giant arcs, and I present here a report of the

developments as seen from Princeton.

The discovery was first announced on television and in the newspapers, so naturally it was difficult for most astronomers at Princeton to know what was happening. A few days later M. Postman (PUO) returned from Pasadena with more specific news, while a photograph of one arc appeared in *Time* magazine. That was sufficient to trigger several speculations, somewhat predictable in that they may reflect the preoccupations of their originators.

As the arcs are superimposed on clusters of galaxies it is natural to think that they are physically associated with each other. J.P. Ostriker (PUO) suggested that the arcs might be a result of powerful explosions. If the central galaxy of a cluster had an active nucleus some time ago, and released a typical energy of the order of 10^{61} – 10^{62} ergs, and there was a lot of cool gas in the cluster of galaxies, then a shock wave would propagate out from the centre of explosion, and it would cool down at a distance of about 100 kpc. Once the spherical shell cooled down it would become gravitationally unstable, and a lot of young stars would be formed. As the arcs do not form complete rings, the gas distribution in the clusters could not be spherically symmetrical. D. Spergel (IAS) proposed that we are seeing a case of galaxy cannibalism, with an arc formed by a galaxy tidally stripped by the cluster.

Over the past few years I have worked on various aspects of gravitational lensing; therefore it was natural for me to propose

that the arcs are two new examples of that phenomenon. The possibility that rich clusters of galaxies act as gravitational lenses has been suggested many times^{3–6}, but so far there is no published and convincing case of such a phenomenon.

It is well known that if a point source is exactly behind a centre of an axially symmetrical lens then its image is a circle, sometimes referred to as the Einstein ring. If the lens has an isothermal mass distribution with its components (that is, stars, galaxies, massinos or anything) having a velocity dispersion v^2 , then the angular radius of the circle is roughly v^2/c^2 , where c is the speed of light. This radius may be as small as 1 arc second if the lens is a medium-sized galaxy with a small velocity dispersion, or as large as 1 arc minute if the lens is a rich cluster of galaxies with a very large velocity dispersion. If the background object is not exactly aligned with the symmetrical lens then we expect to see two arcs on the opposite sides of the lens. A real gravitational lens is not likely to be perfectly symmetrical, and the resulting image of a background galaxy does not have to be a complete ring or a pair of identical arcs on the opposite sides of the lens. Kovner recently presented⁷ some theoretical pictures of a gravitationally lensed galaxy, with highly distorted, multiple images. Some of his pictures display somewhat irregular arc-like structures. It is almost certain that by fine-tuning the structure of the gravitational lens it is possible to obtain images with any desired degree of symmetry.

The giant arcs discovered by Lynds and Petrosian are not complete, structureless rings, but rather arcs with noticeable substructure. It is possible that they are images of galaxies located far behind the clusters. The considerable extent of the arcs implies that we are almost exactly in the focus of the lens system, a very unlikely coincidence. On the other hand, the giant arcs are very rare. Taking complex selection criteria and our poor knowledge of the structure of clusters of galaxies into consideration, it is not possible to make a meaningful estimate of the probability of a coincidence. Fortunately, there is a very straightforward prediction of the proposed explanation: the redshifts of giant arcs must be much larger than the redshift of clusters. The arcs are very faint and their redshifts will be very difficult to measure, but once measured they should provide an unambiguous test of the gravitational lens hypothesis.

It is probably premature to speculate more on the basis of rather limited information available. A very important but difficult observation is needed: the very faint spectra of these luminous, giant arcs. If the redshift of arcs turns out to be larger than the redshift of clusters, then the gravitational lens model will be the most likely. If the redshift of arcs turns out to be the

IMAGE
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REASONS

same as that of clusters, then some process within the clusters must be responsible for their formation. In this case it may be more difficult to discriminate between the models I have mentioned here. The redshift alone will not be enough; other spectral information will be needed. Considering all the excitement it is somewhat surprising that nobody has suggested that superconducting cosmic strings (another novel topic at Pasadena) are somehow related to the giant arcs. □

1. Lynds, R. & Petrosian, V. *Bull. Am. astr. Soc.* **18**, 1014 (1986).
2. Kristian, J., Sandage, A. & Westphal, J.A. *Astrophys. J.* **221**, 383-394 (1978).
3. Paczyński, B. & Górski, K. *Astrophys. J.* **248**, L101-104 (1981).
4. Sanders, R.H., van Albada, T.S. & Oosterloo, T.A. *Astrophys. J.* **278**, L91-94 (1984).
5. Narayan, R., Blandford, R. & Nityananda, R. *Nature* **310**, 112-113 (1984).
6. Turner, E.L., Ostriker, J.P. & Gott, J.R. III *Astrophys. J.* **284**, 1-22 (1984).
7. Kovner, I. *Astrophys. J.* (in the press).

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Igneous geochemistry

Reading radioactive rock clocks

Ross W. Williams

RADIOACTIVE clocks are continuously ticking. In magma source regions, in magma chambers, in minerals crystallized from magmas and after eruption in frozen volcanic effusives, they continue to record time. These clocks are the nuclides of the naturally occurring uranium and thorium radioactive decay series. They are wound by fractionations between the members of the decay series, set when the fractionation process ends and run with characteristic rates determined by the radioactive decay constants of the various isotopes. We are beginning to learn how to read these clocks, as demonstrated by M. Condomines *et al.* on page 607 of this issue¹.

Three naturally occurring radioactive decay series are headed by the very long-lived parents ²³⁸U, ²³⁵U and ²³²Th. Each isotope decays through several intermediate steps, represented by nuclides unique to each decay series, to their final daughters ²⁰⁶Pb, ²⁰⁷Pb and ²⁰⁸Pb, respectively, the stable isotopes of lead. When a decay series is in radioactive equilibrium, the decay rate, or activity, of all the intermediate nuclides is equal to that of the parent. Radioactive disequilibrium occurs when, as a result of fractionation, one of the intermediate daughter isotopes is enriched or depleted with respect to its parent. Once the fractionation process ends, the daughter decays or grows towards equilibrium at a rate proportional to its half-life. The figure shows the rates at which different daughter/parent pairs recover to equilibrium (represented by the dashed line at 1.0) after fractionation ends at time $t=0$.

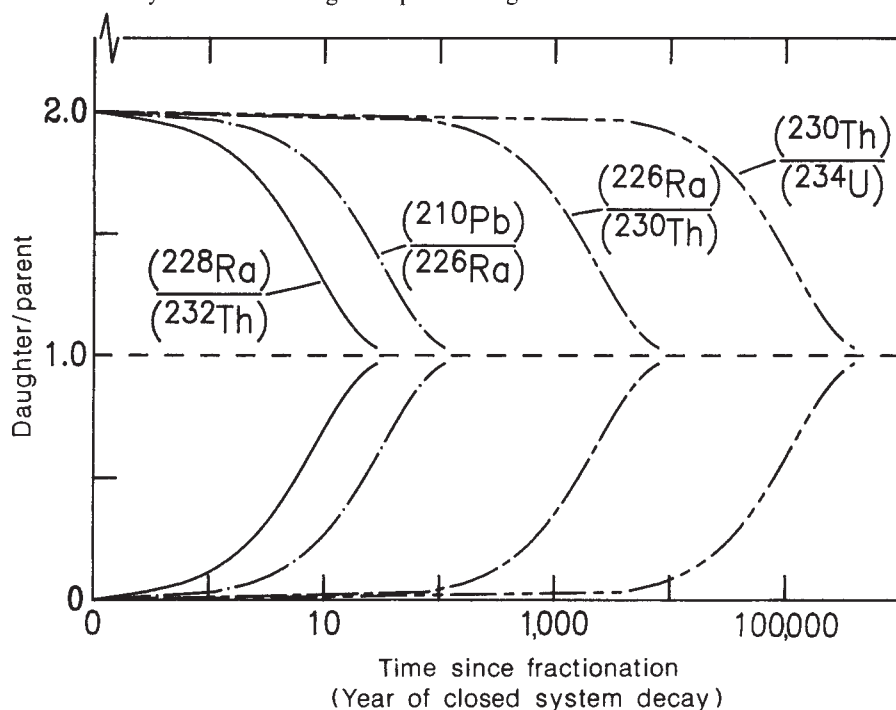
For almost 30 years, radioactive disequilibria between these nuclide pairs have been used for geochronology by marine scientists. Continuing the analogy with the radioactive clocks, igneous geochemistry has lagged behind marine geochemistry because it is not known how tightly these clocks are wound by igneous processes. That is, we do not yet know the magnitude of the fractionations at $t=0$ that can be generated during magma

formation, segregation, differentiation, contamination or eruption. Whereas the fractionating phases in the oceans are directly sampled by marine scientists (for example, sediments/sea water, CaCO₃/sea water), no direct samples exist of a magma source region, its conduits and chambers, or the country rock and fluids with which it may have chemically interacted. Although potential sources (or sinks) of the decay-series elements in magma systems can be sampled by drilling, or sampled by the magma itself through the presence of inclusions in erupted lava, observed disequilibria have been attributed to particular processes largely by inference.

Uncertainty about which magmatic pro-

cesses cause fractionation between the decay-series elements is largely the result of the relatively small dataset available for rocks of diverse chemistry and provenance. As radionuclide data for young volcanics are collected, patterns of enrichment in various rock-types begin to emerge. For example, Condomines *et al.*¹ suggest that radium enrichment is correlated with uranium enrichment, which may reflect magma formation in a wet (H₂O-CO₂ fluid) environment. Growing interest in the decay-series nuclides and a promising new analytical development, measurement of ²³⁰Th and ²³⁴U by mass spectrometry², will expand the dataset. Although no mass-spectrometric measurements of ²³⁰Th and ²³⁴U have been reported for volcanic rocks, their analysis could become routine for researchers with high-resolution, solid-source mass spectrometers equipped with appropriate detectors for small ion currents. Only the most abundant (longest-lived) daughters of the ²³⁸U decay chain can be analysed by this method. The activities of the shorter-lived members of the ²³²Th and ²³⁵U decay series will continue to be measured by radiometric counting techniques.

Two of the central questions in igneous geochemistry that study of radioactive disequilibria can help to answer are: what are the rates of magma genesis; and what are the timescales of magma separation and transport? In addition, radionuclide data can help to resolve other questions about processes that occur during the ascent of a magma. These include differentiation by



The curves show the paths that selected daughter/parent activity ratios will follow with time after a fractionation event at $t=0$. Two sets of initial conditions are shown for each nuclide pair: (1) a twofold enrichment of the daughter over the parent (upper traces); and (2) no daughter present at $t=0$ (lower traces). The time required to reach equilibrium is proportional to the half-lives of the daughters. For each pair, the curves end at $t=5$ times the daughter's half-life ($\sim 97\%$ ingrown).

solid/liquid, liquid/liquid or liquid/vapour fractionation mechanisms; and contamination, which can include mixing with other magmas and assimilation of wall-rock or foreign fluids.

This research promises to increase our understanding of the physiochemical nature of igneous processes, as well as the timescales over which they operate. Researchers have begun to use the radioactive clocks to see into the mantle of the Earth where magmas are generated. D. McKenzie³, interpreting the $(^{230}\text{Th})/(^{238}\text{U})$ data for mid-ocean ridge basalt^{4,5} by a dynamical melting model for genesis of this basalt, has suggested that "the melting occurs over a time long compared with the decay time of ^{230}Th , and that less than 2 per cent of the rock is molten at any time". Although an important contribution, McKenzie's interpretation can be challenged because of the restricted values for the solid/liquid distribution coefficients of uranium and thorium which he con-

sidered. As these distribution coefficients are more precisely determined, however, decay-series activity data will help to constrain partial melting processes and their relative timescales.

Integrating radionuclide data with other isotope and trace-element data is a powerful method for investigating igneous processes that cannot be fully understood by any one of these methods separately. The radioactive clocks will therefore become an indispensable tool for igneous petrologists involved in the study of magma dynamics □

1. Condomines, M. *et al.* *Nature* **325**, 607-609 (1987).
2. Edwards, R.L., Chen, J.H. & Wasserburg, G.L. *Trans. Am. geophys. Un.* **67**, No. 44, 1248 (1986).
3. McKenzie, D. *Earth planet. Sci. Lett.* **72**, 149-157 (1985).
4. Condomines, M., Morand, P. & Allegre, C.J. *Earth planet. Sci. Lett.* **55**, 247-256 (1981).
5. Newman, S., Finkel, R.C. & Macdougall, J.D. *Earth planet. Sci. Lett.* **65**, 17-33 (1984).

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Molecular medicine

Oncogenes in atherosclerosis

James Scott

ATHEROSCLEROSIS, a disorder of large arteries that develops slowly over many years, is the cause of coronary heart disease and cerebrovascular disease. The lesions of atherosclerosis occur in the innermost layer of the affected arteries and consist of plaques of proliferated smooth muscle cells infiltrated with macrophages and embedded in a matrix of basement membrane, proteoglycan and connective tissue. Many of the cells in this lesion have taken up cholesteryl esters derived from low-density lipoprotein (LDL) and their cytoplasm has a characteristic foamy appearance. The most popular theory¹ for the pathogenesis of these atherosclerotic plaques is that they develop as a consequence of chronic cycles of vascular wall injury and repair, and that injury is exacerbated by risk factors for atherosclerosis such as hypercholesterolaemia, hypertension and smoking cigarettes. An alternative and less-fashionable view^{2,3}, based on the observation that atherosclerotic plaques have monoclonal character for isoenzymes of glucose-6-phosphate dehydrogenase (G-6-PD), is that each plaque represents a benign neoplasm derived from a cell that has been transformed by viruses or chemicals. The recent demonstration⁴ by Penn and colleagues that human atherosclerotic plaques contain active oncogenes gives new impetus to this 'monoclonal' hypothesis.

Pathological studies in man show that development of the fatty, fibrous atherosclerotic plaque is preceded by the fatty streak, a subendothelial accumulation of

cholesterol-engorged macrophages and smooth muscle cells. Animals that are fed cholesterol show a similar progression from fatty streak to fibrous plaque, with macrophage adherence to and infiltration of the endothelium being significant early events¹. Later, there is endothelial retraction over fatty streaks and formation of platelet thrombus; ultimately smooth muscle cells and matrix components accumulate to result in a fibrous plaque. Each cellular element involved in the pathogenesis of the atherosclerotic plaque harbours platelet-derived growth factor (PDGF), the major mitogen for vascular smooth muscle cells^{1,5}. PDGF is a basic protein of relative molecular mass 30,000 with two disulphide-linked chains (A and B) and is active as a homodimer of A or B and as a heterodimer of A and B chains. It is also a chemoattractant for smooth muscle cells and stimulates the secretion matrix components from them. The most abundant source of PDGF is the platelet α -granule, the constituents of which are released during platelet adherence to damaged vascular endothelia. Apart from platelets, it has recently been shown^{3,6} that PDGF B chain is produced by macrophages and endothelial cells and that the A chain is secreted by smooth muscle cells in tissue culture. Platelets and macrophages also contain factors that effect endothelial cell mitosis including α - and β -transforming growth factors and fibroblast growth factors^{1,6}. Moreover, smooth muscle cells secrete insulin-like growth factor-I which augments the

mitogenic effect of PDGF on these cells⁶. Taken together, these observations suggest that PDGF, produced by platelets in response to endothelial injury and by macrophages and arterial wall cells in response to more subtle damage, leads to the recruitment and proliferation of smooth muscle cells and to the secretion of the matrix components that cause the atherosclerotic plaque¹.

It is well known that human atherosclerotic plaques have monoclonal character for isoenzymes of G-6-PD (refs 2,7,8). The gene encoding G-6-PD resides on the X chromosome so that, in accord with the Lyon hypothesis⁹, one copy is inactivated randomly during development in females. As a consequence most tissues form a mosaic of patches derived from cells in which one or other X chromosome has been inactivated. The patches are generally small so that representative samples of tissue in heterozygous individuals will contain both isoenzymes of G-6-PD. The finding of a high frequency^{2,7} of monoclonality for G-6-PD in atherosclerotic plaques led to the suggestion that the plaque is a primary benign neoplasm². But Thomas *et al.*⁸ found the monoclonal character for G-6-PD in only 30 per cent of the many lesions they investigated. The explanations they considered include secondary selection of smooth muscle cells that have undergone mutation, producing a selective growth advantage; the phenotypic selection of cells containing one or other of the isoenzymes of G-6-PD resulting from the remote presence on the X chromosome of an allele that confers advantage; or more simply that the monoclonal character of plaques is caused by sampling of plaques originating from a single patch of the aortic mosaic.

The work of Penn and colleagues⁴ provides further support for the monoclonal hypothesis. They demonstrate the presence of an active human oncogene in plaques by transformation of cultured mouse fibroblast NIH 3T3 cells with transfected DNA from plaques. The transformed cells could establish tumours in 'nude' mice, but unlike the aggressive growth produced by the *ras*, *met* and *neu* oncogenes, of which *ras* was excluded by hybridization, the tumours produced by plaque DNA have a slow growth rate and low tumour incidence. No transforming potential is elicited from normal arterial wall DNA. These studies therefore implicate mutation leading to activation of one or more dominantly active oncogenes in the cells that make up the atherosclerotic plaque—a process demonstrated previously only in cancer cells.

If the observations of Penn *et al.* can be confirmed, they raise important questions concerning the stage in the pathogenesis of the atherosclerotic plaque at which oncogene activation occurs and the cell type that is transformed. Although they

did not examine fatty streaks, these lesions do not apparently show monoclonality⁷. The fatty streak does, however, provide an environment with significant mutagenic potential in which oncogene activation might occur and lead to plaque development. Chronic inflammation certainly predisposes to neoplasia and transformation can be produced by human phagocytes stimulated to produce lipid peroxides, reactive oxygen intermediates^{10,11}. In addition, macrophages and endothelial cells modify LDL by free-radical attack with the peroxides^{12,13} and the core of LDL may act as a sink for lipid-soluble mutagens. Significant amounts of modified LDL may be taken up by macrophages and smooth muscle cells during generation of foam cells, predisposing them to transformation.

What is the active oncogene in the atherosclerotic plaque? PDGF is an obvious candidate because its B-chain gene is almost identical to the transforming gene of the simian sarcoma virus¹⁶. This virus transforms many PDGF-responsive cells in tissue culture, including smooth muscle cells, fibroblasts and NIH 3T3 cells, and the phenotype of virus-transformed cells is relatively benign, being intermediate between that of normal cells and that produced by Kirsten mouse sarcoma virus. Many cells transformed in culture by agents other than simian sarcoma virus or obtained from naturally occurring tumours produce a PDGF-like protein. It is not clear whether PDGF produced in these ways contributes to the neoplastic process by an autocrine mechanism or whether it has a paracrine effect that recruits mesenchymal cells into the tumour mass. But these observations may be important in relation to atherosclerotic plaque formation. If proto-oncogene activation leads to increased secretion of A and/or B chains as either a primary or a secondary mechanism, there would certainly be increased smooth muscle growth within the arterial wall. □

- Ross, R. *N. Engl. J. med.* **314**, 488-500 (1986).
- Benditt, E.P. & Benditt, J.M. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1753-1756 (1973).
- Benditt, E.P., Barrett, T. & McDougall, J.K. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6386-6389 (1983).
- Penn, A. Garte, S.J., Warner, L., Nesta, D. & Mindich, B. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7951-7955 (1986).
- Sejersen, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 6844-6848 (1986).
- Ross, R., Raines, E.W. & Bowen-Pope, D.F. *Cell* **46**, 155-169 (1986).
- Pearson, T.A., Wang, A., Solez, K. & Heptinstall, R.H. *Am. J. Path.* **81**, 379-387 (1975).
- Thomas, W.A., Reiner, J.M., Janakidevi, K., Florentin, R.A. & Lee, K.T. *Expl. molec. Path.* **31**, 367-386 (1979).
- Lyon, M.F. *Am. J. hum. Genet.* **14**, 135-148 (1962).
- Weitzman, A.B., Weitzman, S.A., Destremes, M. Latt, S.A. & Stossel, T.P. *N. Engl. J. Med.* **308**, 26-30 (1983).
- Weitzman, S.A., Weitzman, A.B., Clark, E.P. & Stossel, T.P. *Science* **227**, 1231-1233 (1985).
- Parthasarathy, S., Printz, D.J., Boyd, D., Joy, L. & Steinberg, D. *Arteriosclerosis* **6**, 505-510 (1986).
- Wolff, S.P., Garner, A. & Dean R.T. *Trends biochem. Sci.* **11**, 27-31 (1986).

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X-ray scattering

A new probe of protein dynamics

Peter Artymiuk

THE importance of the dynamic aspects of the behaviour of biological macromolecules is now widely recognized. In many areas of molecular biology, such as muscle contraction or conformational changes in allosteric proteins, motion is self-evidently crucial. Again, in facilitating such processes as the access of ligands to binding sites on proteins, or in accommodating the change in protein and substrate geometry associated with the execution of an enzymic reaction, coordinated molecular motion is often essential. Knowledge of dynamic processes in proteins initially came from spectroscopic methods such as fluorescence quenching and nuclear magnetic resonance, and has been increased by theoretical molecular-dynamics simulations¹. The spectroscopic methods give very local dynamic information concerning only a few of the amino-acid residues in a protein. More recently, analyses^{2,3} of the isotropic atomic temperature factors, obtained from crystallographic refinement against the Bragg diffraction intensities from protein crystals, have provided an overall view of the amplitudes of displacements of atoms throughout complete protein structures. A further step towards the understanding of protein dynamics in the crystal is described on page 643 of this issue⁴, where Doucet and Benoit report on one aspect of the diffuse, non-Bragg scattering of X-rays from crystals of lysozyme.

Two types of X-ray diffuse scattering can be distinguished⁵. The first is thermal diffuse scattering, which concerns modes of vibration and elasticity in the crystal. It arises from correlated displacements of atoms throughout the crystal, and is manifested as diffuse spots around the positions of the Bragg peaks. In principle, the positions, shape and intensities of these thermal diffuse maxima contain information about the direction, location and extent of correlated displacements. Extracting this information would provide invaluable data about concerted motions of groups of atoms within protein molecules. The second kind of diffuse scattering results from disorder and is attributable to crystal imperfections.

It is this latter aspect of the diffuse scatter that concerns Doucet and Benoit. These authors demonstrate⁴ the existence of correlated rigid-body displacements of groups of lysozyme molecules in a crystal. Clearly, these displacements are just a function of the particular crystal form of lysozyme studied, and are not of biological significance. But because the rewards of more complete analyses of dif-

fuse scattering would be so great, the emergence of methods for measuring and modelling the diffuse scattering is very important. It is to be hoped that the methods used by Doucet and Benoit will be extended to include thermal diffuse scattering, perhaps through the use of molecular-dynamics simulations⁷. One example of the analysis of X-ray diffuse scattering from protein crystals to yield biologically pertinent data already exists: Phillips *et al.*⁶ have investigated diffuse scattering from tropomyosin crystals and attempted to relate the crystalline disorder to the movement of tropomyosin during regulation of muscle contraction.

In principle, the information that could be obtained from diffuse scattering would substantially augment that obtainable from crystallographic temperature factors: there would be the possibility of describing large displacements of whole subunits or domains in proteins; the presence or absence of cooperativity between different displacements could be established; and the directionality of displacements could be defined without the requirement, necessary for anisotropic temperature-factor refinement, of unusually strong diffraction at high angles.

There is, however, a major deficiency in the information about dynamics available from both X-ray diffraction and X-ray diffuse scattering: both supply time- and space-averaged accounts of the distribution of matter within the crystals and give no information about the timescales of the displacements they detect. Indeed, the displacements discovered may not be dynamic at all, but may result from static disorder in the crystal. There have been attempts to discriminate between static and dynamic disorder by doing experiments at different temperatures², but because dynamic disorder can 'freeze' into static disorder at low temperature, such studies may not be conclusive. In thermal diffuse scattering, authentic dynamic effects cause a tiny change in the wavelength of the scattered X-ray as energy is inelastically transferred to quantized lattice vibrations (phonons) in the lattice, but this change is too small to be measured. Inelastic neutron scattering has been used to demonstrate⁸ the dynamic origins of thermal diffuse scattering in β -quartz, but has yet to fulfill its promise in probing protein dynamics. At this stage in our understanding of motion in biological macromolecules, however, data on conformational variations, whether they happen to be static or dynamic in the crystal, are valuable indicators of the in-

herent flexibility of protein molecules.

But does the behaviour of protein molecules inside a crystal have any relevance to the dynamic properties of protein molecules in solution? Petsko and Ringe⁹ have reviewed this question and the evidence (which includes studies of enzymic catalysis and of hydrogen-deuterium exchange in the crystal) suggests that the answer is often yes. The reason is that typically 50 per cent of the volume of a protein crystal consists of wide expanses of the solvent of crystallization (usually bulk water or an aqueous salt solution), with relatively few actual contacts between protein molecules. Although crystal contacts can have a damping effort on the atomic displacements¹⁰, it is frequently found that large-scale conformational variability can be accommodated within protein crystals. In the crystals of immunoglobulin IgG, for example¹¹, the entire F₂ domain (a region of relative molecular mass 50,000) is disordered and cannot be seen in the electron-density maps. Such cases of disorder must be related to the inherent flexibilities of the protein molecules concerned, which in turn may be related to their function.

In the past few years, the use of high-intensity synchrotron radiation for crystallographic data collection has shown that many protein crystals exhibit diffuse scattering maxima. The study of these potentially very informative adjuncts to diffraction patterns is only just beginning. Nevertheless, it is to be hoped that before long, analysis of X-ray diffuse scattering from such crystals will reveal much more about cooperative motions and conformational variability in protein molecules. □

1. Karplus, M. & McCammon, J.A. *A. Rev. Biochem.* **53**, 263-300 (1983).
2. Frauenfelder, H., Petsko, G.A. & Tsernoglou, D. *Nature* **280**, 558-563 (1979).
3. Artymiuk, P.J. *et al. Nature* **280**, 563-568 (1979).
4. Doucet, J. & Benoit, J.P. *Nature* **325**, 643-646 (1987).
5. Guinier, A. (translated by Lorrain, P. & Lorrain, D. S.-M.) *X-ray Diffraction in Crystals, Imperfect Crystals and Amorphous Bodies* (Freeman, San Francisco, 1963).
6. Phillips, G.N. Jr, Fillers, J.P. & Cohen, C. *Biophys. J.* **32**, 485-502 (1980).
7. Helliwell, J.R. *et al. Biochem. Soc. Trans.* **14**, 653-655 (1986).
8. Bauer, K., Jagodzinski, H., Dörner, B. & Grimm, H. *Phys. Stat. Sol.* **B48**, 437-443 (1971).
9. Petsko, G.A. & Ringe, D. *A. Rev. Biophys. Bioengng.* **13**, 331-371 (1984).
10. Finzel, B.C. & Salemme, F.R. *Nature* **315**, 686-688 (1985).
11. Colman, P.M., Deisenhofer, J., Huber, R. & Palm, W. *J. molec. Biol.* **100**, 257-282 (1976).

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Reinhold Benesch (1919 – 1986)

SOME people are said to be accident-prone. Reinhold Benesch, who died in New York on 30 December 1986, was discovery-prone. His first piece of research was a contribution to the British war effort. When asked to develop a way of packing eggs for the troops more tightly, he fed hens with sulfanilamide, which inhibited the enzyme carbonic anhydrase that is essential for calcification of their eggshells; the shell-less 'floppy' eggs could be packed like matchboxes. Benesch never told me whether the army adopted his invention. Once he sent me a reprint of a paper entitled *Homos and Heteros Among the Hemos* inscribed "With cordial greeting R'B". The paper was about homo- and hetero-oligomers of haemoglobin and the signature symbolized the 'heterodimer' Ruth and Reinhold Benesch. They got married in England in 1946, emigrated to the United States in 1947 and for the past 26 years worked together at the Columbia College of Physicians and Surgeons. They became the joint authors of 125 papers, all but 13 on haemoglobin. Ruth's contribution to them is inseparable from Reinhold's.

Oxygen equilibrium curves of purified human haemoglobin solutions differ from those of haemolysates. This made the Cambridge physiologist Joseph Barcroft suspect as early as 1921 the presence of a "third substance ... which forms an integral part of the oxygen haemoglobin complex". Years later, it also made his pupil F.J.W. Roughton dismiss my X-ray work on pure haemoglobin crystals as physiologically irrelevant. In 1967 the Benesch

discovered that Barcroft's third substance is 2,3-diphosphoglycerate (DPG), long known to be present in erythrocytes in equimolar proportion to haemoglobin. That discovery opened a new era in the physiology of respiratory carriage. Some of the best among the ensuing flood of papers are those by the Benesch themselves. They demonstrated that one mole of DPG combines with one mole of deoxy-, but not with oxyhaemoglobin and that DPG lowers the oxygen affinity in a physiologically advantageous way. They determined the thermodynamics of DPG binding; its influence on the Bohr effect and its interactions with other solutes; they also foreshadowed its location in the central cavity between the β -chains which A. Arnøne later determined by X-ray analysis. Subsequent work by several investigators indicated that DPG also binds weakly to oxyhaemoglobin and that its more powerful analogue inositol hexaphosphate (IHP) binds to oxyhaemoglobin quite strongly. Judging by chemical evidence, DPG and IHP appeared to bind to the same residues as in deoxyhaemoglobin, even though the cleft where they bind is closed in oxyhaemoglobin. I tried to solve that paradox by X-ray analysis, but drew a blank. The Benesch invented an ingenious method for measuring the dissociation constants of weakly bound ligands and used it to demonstrate that DPG and ATP, and by implication also IHP, bind, not to oxyhaemoglobin tetramers, but to $\alpha\beta$ -dimers, where the residues that are tucked away in the cleft of the tetramer are exposed. The satisfying

answer to this long-standing puzzle forms the subject of the Benesch's last, and perhaps most elegant, joint paper.

On several other occasions their work brought light where there had been darkness. The oxygen equilibrium curves of human haemoglobin measured by different workers used to be inconsistent, because their solutions contained variable amounts of DPG and they used anionic buffers which bound to deoxyhaemoglobin and reduced the oxygen affinity in varying degrees. The Benesch developed methods of stripping away the DPG and introduced his Tris buffer which has a pK of 6.5 and is mostly uncharged at physiological pH, so that it is not bound by deoxyhaemoglobin. Only since then have oxygen equilibria become reproducible. They started their first work on haemoglobin in response to reports that its SH groups contribute to the Bohr effect. The Benesch disproved this, even though they found that the SH reagent N-ethylmaleimide halves the alkaline Bohr effect. That discovery led me to examine the N-ethylmaleimide derivative by X-ray analysis, which revealed that this reagent inhibits formation of a salt bridge formed by the carboxy-terminal histidines of the β -chains and thus led to the first interpretation of a physiological function of haemoglobin on a stereochemical basis. The paper on homos and heteros among the hemos demonstrated that tetramers of β - and γ -chains, which undergo no change in quaternary structure, bind oxygen non-cooperatively, thus proving that a heterodimer undergoing a structural transition is essential for cooperativity.

On loss of oxygen, sickle-cell haemoglobin polymerizes into long fibres. Knowledge of the contacts between the molecules in the fibres would facilitate the design of anti-sickling drugs. The Benesch explored these contacts by making hybrids of β -chains carrying the sickle-cell mutation with a variety of mutant α -chains. They found their effects to be highly specific: some mutations had no influence on the solubility of deoxyhaemoglobin S; some raised it; one lowered it and changed the fibre geometry. The results enabled them to map out a hitherto unrecognized contact area. Recently they discovered that folate, in its poly- γ -glutamate complex, is carried in the blood by haemoglobin to which it binds at the same site as DPG.

Reinhold Benesch was a first-class biochemist and also an accomplished wit and comedian who brought laughter to the dreariest of scientific meetings. His inventiveness and originality shone in his papers and in discussion; his wide literary knowledge and *joie de vivre* made him a delightful companion on non-scientific occasions.

Max Perutz

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Atomic physics

New patterns in atomic spectra

A. R. P. Rau

CAN there still be anything new in the problem of a hydrogen atom in a uniform magnetic field? Most physicists are likely to say no and be surprised that this seemingly simple problem is not yet fully understood and that unexpected and qualitatively new phenomena continue to emerge. Recent experiments^{1,2} by a group at the University of Bielefeld provide the latest twist to a fascinating story that has been developing over the past fifteen years³⁻⁵. The story is a good example of how new patterns and structures, whose understanding poses non-trivial questions, can emerge from combining two well-understood forces. This simple atomic system seems to be rich in its implications for the general study of processes such as non-separable and non-integrable classical and quantum mechanics, and the nature and onset of chaos.

The motion of an electron in the Coulomb field ($-e^2/r$) of a positive charge or in the diamagnetic potential ($\frac{1}{2} B^2 \rho^2$, where $\rho^2 = x^2 + y^2$) of a uniform magnetic field B (along the z -direction) is a familiar one when either field acts alone. The Coulomb problem has an infinite Rydberg progression of bound-state energy levels that terminate at the edge $E = 0$ of the continuum; the higher energy levels ($E \lesssim 0$) are highly degenerate. The magnetic problem, on the other hand, has an

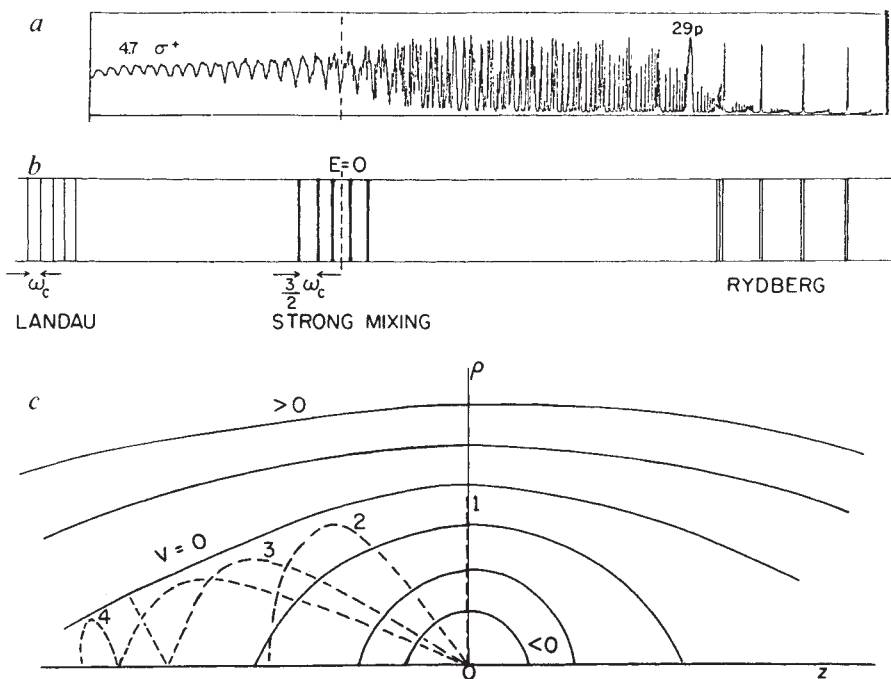
infinity of equally spaced energy levels for the motion in x and y (the Landau spectrum, with spacing equal to $\hbar\omega_c$, where ω_c is the cyclotron frequency and $\hbar$ is Planck's constant) and free motion along z . Once again each Landau level is highly degenerate. When both fields act together, the situation is again familiar when the magnetic field is weaker than the Coulomb field. Perturbation theory then applies for the diamagnetic interaction or for the linear coupling of the magnetic moment of the electron to the magnetic field (Zeeman and Paschen-Back effects). The spectrum is a Rydberg pattern with some sub-structure imposed by the magnetic field on each energy level. Similarly, in the past fifteen years, in dealing with atomic structure on neutron stars (believed to have large magnetic fields, $B \sim 2 \times 10^8$ tesla), there have been extensive studies⁶ of the regime when the diamagnetic potential is dominant and the Coulomb field secondary. Again, standard techniques apply and the spectrum can basically be described as equally spaced Landau levels (spacing ~ 10 keV) with sub-structure caused by the Coulomb potential.

But what happens in between, when neither field can be considered perturbative and both terms in the Coulomb-diamagnetic potential, $V(\rho, z) = (-e^2/r)$

+ $\frac{1}{2} B^2 \rho^2$ (where B is in units of 4.7×10^5 tesla), are equal. For any value of B , this situation will obtain around $E = 0$. Starting with the basis of eigenstates of either field alone, an infinite number of states are mixed together by the full potential. One may then expect no simple regularities or structures in this "strongly mixed"³ region of the spectrum. Yet in 1968 the first experimental results from this region belied this expectation. The figure (a) shows an absorption spectrum⁷ of the principal series in strontium in a field of $B = 4.7$ tesla. The most striking feature of this and subsequent experiments with other atoms is the pattern of broad, equally spaced resonances around $E = 0$. Regardless of the atom, the spacing between resonances is $(3/2)\hbar\omega_c$.

Therefore, as shown in *b*, the problem of combined Coulomb and diamagnetic motions seems to have three, not two, regimes with characteristic signatures. Complementing the two signatures of Coulomb and Landau patterns, when one or the other field is dominant, is the equally characteristic signature of the strong-mixing regime³. A full understanding of the origins of a simple pattern in a highly non-perturbative regime and the generality of this result (similar results obtain for strong mixing of Coulomb and electric fields⁸) are still open questions. The new results^{1,2}, the first experiments with hydrogen, hold another surprise. As well as the $(3/2)\hbar\omega_c$ resonances, there are other families of equally spaced resonances with other spacings.

The Coulomb-diamagnetic potential $V(\rho, z)$ is non-separable and not exactly solvable. A partial understanding of the experimental observations can be gained from the equipotential lines sketched in *c*. After the initial experiments, it was realized^{8,9} that in considering the motion of an electron on this equipotential surface, if one envisages motion along the ρ -axis, then for every energy E (even $E > 0$) this motion is bounded. The electron has both ρ - and z -motions, the two are coupled by the Coulomb term in $V(\rho, z)$ so that for any $E > 0$ the latter motion will eventually lead to escape. But, application of a semi-classical quantization condition for the path 1 gives^{3,8,9} for $E \sim 0$ a set of energy values with equal spacing of $(3/2)\hbar\omega_c$. The new spacings seen in the hydrogen experiment have now been similarly correlated² with the other paths in *c* through both classical trajectory calculations for recurrence times following successive reflections from the $V = 0$ and $\rho = 0$ lines, and corresponding semi-classical quantization along the paths shown^{2,10}. One can expect decreasing intensity with increasing length of paths. This fits with the $(3/2)\hbar\omega_c$ spacing being dominant in nearly all experiments to date, except one¹ when the vanishing of the wavefunction along the ρ -axis dis-



a, Absorption spectrum of strontium with $\sigma + (m=1)$ polarization in a 4.7-tesla magnetic field (from ref. 7). b, Schematic drawing of a to emphasize three characteristic regimes. c, Equipotentials of Coulomb-diamagnetic potential. The numbered paths correspond to different modes of quantization around $E = 0$.

favours it. It is also clear from *c*, as noted by the Bielefeld group², that for the higher-numbered paths, the higher bounces at large *z* take place mainly under the diamagnetic influence, the Coulomb term being insignificant; therefore, successive recurrence times are staggered by the cyclotron period $T_c = 2\pi/\omega_c$.

There is as yet little progress in going beyond such simple one-dimensional semi-classical calculations for energy spacings. A fuller understanding of the quantum mechanics of the non-separable potential still eludes us. Intensities and widths of the observed strong-mixing patterns remain unexplained.

The Coulomb-diamagnetic problem has also become of interest as a real physical system that is amenable to experiments in which questions of classical and quantum chaos can be studied. Classical trajectory calculations show a passage from regular patterns in phase space to a random chaotic distribution as the $E = 0$ limit is approached from negative energies. Similarly, large-scale numerical diagonalizations of $V(\rho, z)$ in a basis of Coulomb wavefunctions show that the spacing between eigenvalues has a Poisson distribution so long as $B^2/|E|^3$ is small but changes to a Wigner distribution as this parameter becomes large^{12,13}. Given the infinite pile-up of Rydberg levels at $E = 0$, all such calculations have performed a little short of the ionization limit.

Clearly, much is still unknown about this simple but rich atomic system. There

is already an indication of further surprises in store because the hydrogen experiment shows other spacings that cannot be correlated with the sequence of paths in *c*. But it is also clear that more experimental and theoretical understanding of this fascinating system of hydrogen in a magnetic field will have wider application for the physics of non-separable potentials. Just as the development of perturbation theory through the study of the Zeeman and Stark effects has contributed tools for general theoretical physics, so also a fuller elucidation of strong mixing in this system is likely to be of interest elsewhere in physics. □

- Holle, A. *et al.* *Phys. Rev. Lett.* **56**, 2594-2597 (1986).
- Main, J., Wiebusch, G., Holle, A. & Welge, K. H. *Phys. Rev. Lett.* **57**, 2789-2792 (1986).
- Rau, A. R. P. *Phys. Rev. A* **16**, 613-617 (1977); *J. Phys.* **B12**, L193-L198 (1979).
- Kleppner, D., Littman, M. G. & Zimmerman, M. L. in *Rydberg States of Atoms and Molecules* (eds Stebbings, R. F. & Dunning, F. B.) 73-116 (Cambridge University Press, 1983).
- Gay, J. C. in *Progress in Atomic Spectroscopy, Part C* (eds Beyer, H. J. & Kleinpoppen, H.) 177-246 (Plenum, New York, 1984).
- Garstang, R. H. *Rep. Progr. Phys.* **40**, 105-154 (1977).
- Garton, W. R. S. & Tomkins, F. S. *Astrophys. J.* **158**, 839-845 (1969).
- Edmonds, A. R. *J. Physique* **31**, C4 71-74 (1971).
- Starace, A. F. *J. Phys.* **B6**, 585-590 (1986).
- Al-Laihy, M. A., O'Mahony, P. F. & Taylor, K. T. *J. Phys.* **B19**, L773-L777 (1986).
- Fano, U. *Atomic Physics* (eds Lindgren, I. & Svanberg, S.) Vol. 8 5-22 (Plenum, New York, 1983).
- Wintgen, S. & Friedrich, H. *Phys. Rev. Lett.* **57**, 571-574 (1986).
- Delande, D. & Gay, J. C. *Phys. Rev. Lett.* **57**, 2006-2009 (1986).

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Neurobiology

Disappearing developing cells

R. W. Guillery and H. P. Killackey

THE early formation of nerve cells and connections, produced in excess of adult needs and made with many 'errors', has over the past 30 years become an acceptable concept to neurobiologists¹. Such regressive phenomena are believed to contribute to the formation of an accurately connected nervous system. The possibly related phenomenon of transient nerve cells, which are present in early development but have no adult counterparts, has been raised in the past, but Chun, Nakamura and Shatz on page 617 of this issue² report new evidence for a population of cortical neurons that establishes synaptic connections and subsequently disappears. This observation suggests that regressive phenomena such as cell death are involved in processes other than error correction during normal neuronal development.

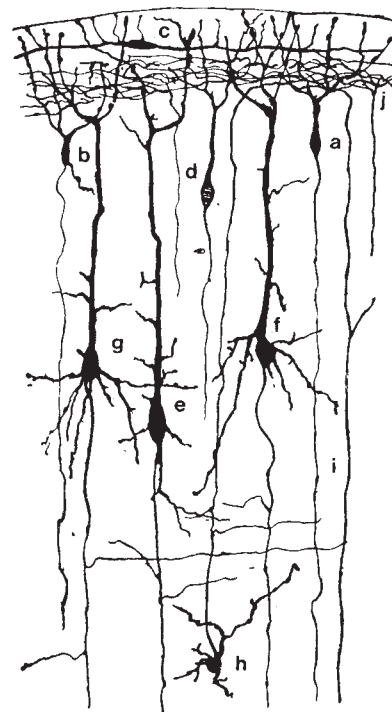
The population of cortical cells reported by Chun *et al.* lies in the cortical subplate; the cells are born early, have local dendrites and longer axons, receive

synaptic connections, develop putative transmitters and then largely disappear during early postnatal development. These cells probably correspond to the Martinotti cells of deep cortical layers (see figure) and the Cajal-Retzius cells of superficial cortical layers, both of which are also formed very early. The disappearance of these cells has long been suspected but it has never before been clear whether they became modified into other cell types or degenerated; nor was it known whether at early developmental stages they had established synaptic connections. By selectively labelling the oldest cortical nerve cells using pulse labels of radioactive thymidine, Chun *et al.* now show that the earliest-formed cortical cells fail to survive even though they develop all the neural characteristics listed above.

What are these neurons doing? They are found before the major cell layers (II-V) of the cortex have developed, and occur simultaneously with the entry of the thalamocortical and interhemispheric

afferents into the cortical subplate region³. Their axons pass to deep cerebral structures and to the opposite hemisphere². Although the cells are immunoreactive for neuropeptides it is not yet known whether these peptides are transported down the axons or whether the axons establish synaptic contacts. Electron-microscope evidence, however, shows that the cells do receive synaptic contacts.

If the cells turn out to be electrically active at this early stage then one can suppose that the temporary impulse traffic is involved in the further development of cortical and subcortical circuitry; the cells are unlikely to play a role in the transmission of sensory or motor functions of the organism at this stage. If they are not discharging impulses then they are probably



Ramon Y Cajal's drawing⁸ of cortex from a four-day-old mouse. This Golgi preparation shows the Martinotti cells (h) and the Cajal-Retzius cells (c) in the cortex at a stage later than that described by Chun *et al.*², after the intermediate cell layers have developed. See text for further details.

concerned with local interactions at each end of the long axonal process; these interactions may be temporary trophic effects.

One such interaction could be the maintenance of a cell population which later acquires a different postsynaptic target, such as the thalamic cells whose axons have reached the cortex at this stage but are 'waiting' in the subplate. The transient cells may provide a temporary target for these axons, because the cortical cells that they will eventually innervate do not develop until much later. The evidence that the transient cells receive contacts with all the morphological appearance of an adult synapse may make morphologists

reluctant to think of the cells as electrically inactive. If nerve cells can make contacts that look like synapses, at which no electrical interaction occurs, then the morphologist is bound to question the significance of the features that are classically considered diagnostic of a synapse.

The transient cells may also serve as tropic markers or 'guideposts' providing a telencephalic equivalent to the guidepost cells defined for developing pathways in other nervous systems⁴. On either hypothesis, however, the function of the axons remains obscure. The long intercerebral pathway is particularly difficult to understand. If it provides a precursor of callosal pathways, why are such precursors needed?

A further possibility is that the transient cortical cells represent the exploitation during mammalian development of cells surviving in the adult of pre-mammalian forms⁵. This would account for the origin, but not for the function, of the cells.

The sequence of developmental events producing the circuitry of the adult brain is extraordinarily difficult to understand. Even where the complexities of the adult are understood (there are few such regions: the cerebral cortex is certainly not one) there are no clear ideas about the ways in which specific patterns of connections are formed. Guidepost cells, sub-

strate pathways⁶ or temporary neural projections that are obliterated or pruned and corrected⁷, provide a conceptual crutch for understanding how complex patterns of connectivity could be formed. But temporary measures for carrying instructions that eventually make a brain are like messengers arriving at a general's headquarters — it is useful to see them arriving, and perhaps even to identify them, the course of their journey and their effect on subsequent events, but the outcome of the battle will be most clearly understood if the nature of the message and the instructions given to the messenger (not necessarily distinguishable) are understood. On these subjects developmental neurobiologists must, as yet, remain silent. □

1. Cowan, W.M., Fawcett, J.W., O'Leary, D.D.M. & Stanfield, B.B. *Science* **225**, 1258–1265 (1984).
2. Chun, J.J.M., Nakamura, M. J. & Shatz, C.J. *Nature* **325**, 617–620 (1987).
3. Wise, S.P. & Jones, E.G. *J. comp. Neurol.* **178**, 187–208 (1978).
4. Palka, J. *Trends Neurosci.* **9**, 482–485 (1986).
5. Marin-Padilla, M. *Anat. Embryol.* **152**, 109–126 (1978).
6. Katz, M.J., Lasek, R.V. & Nauta, H.J.W. *Neuroscience* **5**, 821–833 (1981).
7. Innocenti, G.M. *Science* **212**, 824–827 (1981).
8. Ramon Y Cajal, S. *Histologie du Systeme Nerveux de l'Homme et des Vertebres* Vol. 2 (Maloine, Paris, 1911). Reprinted by Consejo Superior de Investigaciones Cientificas, Madrid, 1972.

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100 years ago

BUBBLES in ice are familiar; but their arrangement and progressive development in the process of freezing-over present some points which I do not think have been generally observed. The following facts must be noticed: (1) Ice over deep water invariably contains fewer bubbles of included air and gas than ice formed over shallow water. (2) The upper portion of a coating of ice invariably contains less included air than its lower portion. (3) There is more included air ice formed over water in a small vessel than ice formed over a large body. (4) There is more included air in an entirely frozen mass of ice than in surface ice from a partly frozen vessel. (5) In freezing separately the water from which the first frozen coat of ice had been removed, the ice contained a much larger proportion of included air than either the surface ice or the ice obtained from entirely freezing a body of water.

From *Nature* **35**, 325; 3 February 1887.

on. In topology, two manifolds are considered to be the same if one can be obtained from the other by a continuous deformation. The surface of a sphere, and that of a torus (or doughnut), are two-dimensional manifolds, but they are topologically distinct because the torus has a hole whereas the sphere does not.

One way to detect the hole in the torus is to thread a loop through it. This loop cannot be shrunk continuously down to a single point while remaining on the surface of the torus. In contrast, on the sphere, every loop can be so shrunk. During the nineteenth century, mathematicians were able to find all possible topological types of two-dimensional manifolds. One consequence is that the only two-dimensional manifold on which all loops can be shrunk to a point is a sphere.

Poincaré was interested in the next step: three-dimensional manifolds, which are much more complicated and at one of the great open frontiers of topology. Even the simplest questions remain unanswered, and the most basic is Poincaré's.

The natural three-dimensional generalization of the two-dimensional sphere is what Poincaré called a hypersphere, and is now called a three-sphere. In the hypersphere, as in the ordinary sphere, every loop can be shrunk to a point. Poincaré asked whether this property completely characterizes the hypersphere. If every loop in a given three-dimensional manifold can be shrunk to a point, must that manifold be topologically a hypersphere? Poincaré asked the question in 1904, and nobody knew how to answer it, although it was widely assumed that the answer must be yes. The problem was still open when Régé and Rourke announced their proof.

Their method is technically complicated, but based on familiar ideas (some, indeed, going back to the time of Poincaré). They start with a description of a three-dimensional manifold in terms of a system of links. They assume that every

Topology

The three-sphere strikes back

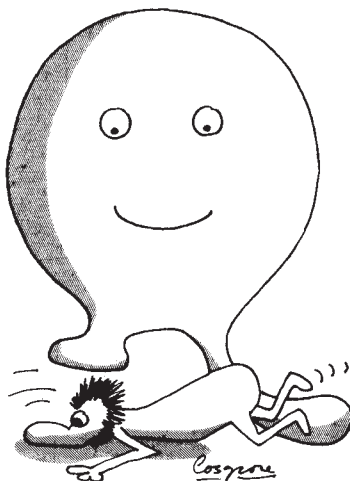
Ian Stewart

ALMOST a year ago I reported in a News and Views article (*Nature* **320**, 217–218; 1986) the announcement, by Eduardo Régé and Colin Rourke, of a proof of the Poincaré conjecture — one of the major unsolved problems of topology. At first, the mathematical community regarded the claim with scepticism. There were good reasons for this. Proofs have been announced before, and retracted soon after, by many distinguished mathematicians. Some thought that Régé and Rourke's methods were not novel enough. Their proof was also long and complicated, difficult to grasp in its entirety and rather sketchy in places. But, for the time being, the sceptics are right.

During the first year, the proof was subjected to thorough scrutiny by the mathematical community. Against most predictions, it held up well until early November 1986. Then Rourke gave a series of seminars to an audience of experts at the University of California at Berkeley, whose objective was to settle the matter once and for all. During a routine discussion, Rourke realized there was a mistake, and pointed it out to the audience. It is in the nature of mathematics that a defective

proof is no proof at all, so the original announcement was wrong. But the story may not be over yet.

The conjecture is about manifolds, which are among the most important objects in topology. Manifolds are spaces which 'in the small' resemble ordinary euclidean space, but which, 'in the large', can bend round upon themselves, twist up in complicated ways, have holes and so



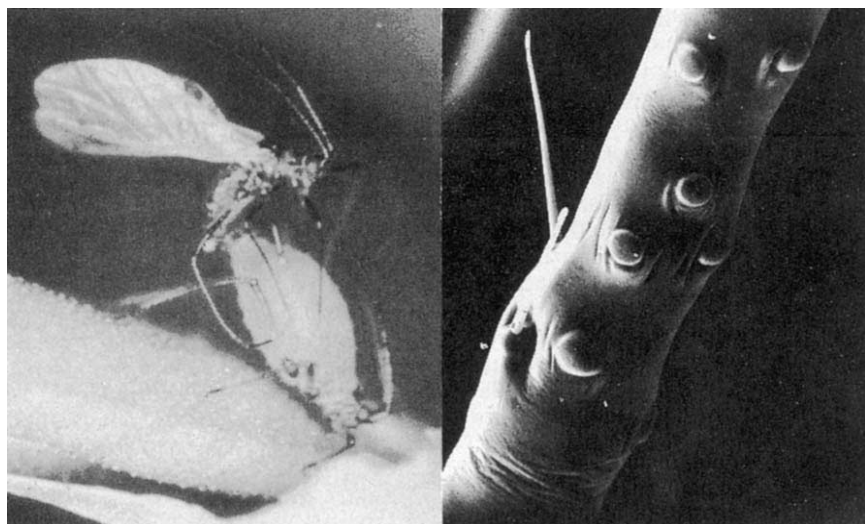
loop in this manifold can be shrunk, and ask what constraints this requirement imposes on the system of links. Then they find ways to modify the links, using those constraints, until at the end of the process they can recognize a system of links corresponding to the hypersphere. Many complicated 'moves' are needed to effect this simplification. Rourke has likened the method to solving the Rubik cube, because this also involves establishing a repertoire of complicated sequences of moves which do useful things.

The sources of technical difficulty are twofold. The first is the combinatorial complexity of the moves, and of the rules stating which one should be applied in which circumstances to achieve which tiny step in the simplification. The second is that, in order for the proof to proceed rigorously, a certain amount of technical baggage must be carried along during each move. At the end of the proof this additional superstructure can be discarded, but until the end is reached it is essential. More than one topologist has attempted to prove the Poincaré conjecture by this route, but nobody had succeeded in fitting all the pieces together and making them all work at once.

Rêgo and Rourke introduced several new ideas relating to the technical baggage, and decided that they had a proof. As far as they were concerned, these new ideas answered the question "what have you got that the others missed?". Ironically, it is one of these pieces of technical baggage that is responsible for the mistake. They impose an "ordering condition" which, roughly speaking, affects the way the various links can interpenetrate. This ordering condition makes some steps in the proof feasible, but it also carries a penalty: after each series of moves, one must check that the ordering condition continues to hold. In fact, it does not hold automatically, but Rêgo and Rourke asserted that the ordering condition could be restored by making some additional moves, which make the proof still more complicated. Unfortunately for them, this assertion seems to be incorrect in at least one case.

How do mathematicians try to avoid such mistakes in logic, and why do mistakes nonetheless occur from time to time? There are three main safeguards. One is to examine every tiny detail of the proof; the danger here is that there are so many details that one escapes attention. The second, which is safer, is to look for internal consistency, and to grasp the overall flow of the proof: if 'everything fits' then there is a fair chance that the underlying ideas are right. The third, and best, is to try out the ideas on lots of simple examples, to see if the results make sense. This is the 'experimental' approach.

The Poincaré conjecture lays several traps for the unwary. The nastiest trap has



Most aphids reproduce parthenogenetically, but some species, such as the vetch aphid *Megoura viciae*, reproduce sexually in winter (left, photograph by G. Higgins). Male vetch aphids locate their mates by detecting an attractive pheromone the females release from their hind legs. The males detect the pheromone with sense organs on the third antennal segment (right). G.W. Dawson *et al.* on page 614 of this issue show that the pheromone contains two components, nepetalactone and nepetalactol. Curiously, nepetalactol gives catmint (*Nepeta cataria* and *N. mussinii*) its characteristic smell and makes it attractive to cats. A mixture of the two compounds is as effective as the authentic pheromone for attracting males. The compounds should be useful for manipulating aphid behaviour, although commercial applications are still a long way off. □

an air of Catch-22 about it. If, as one is trying to prove, the conjecture is true, then the only manifold that satisfies its hypotheses is the hypersphere. Thus the safest method, the 'experimental' one, cannot be used: the only available example is the hypersphere itself, and everything will work automatically on that!

Rêgo and Rourke, having developed their proof over several years, used the second method. To them, the overall flow of the ideas made good sense. They therefore concentrated on explaining this flow. In the process, they suppressed some (supposedly) routine and tedious details, a common practice in mathematical exposition, but one that always carries a certain danger.

Topologists trying to read the proof, equally reasonably, worried more about the fine details. One object of the Berkeley seminars was to fill out those routine details and check that they really were routine. Although it may be small consolation to Rêgo and Rourke, it is a tribute to the topological community that it examined the proof in enough detail to locate the error. The mathematician's natural suspicion, yet again, was vindicated.

The tale also illustrates the differences between mathematical arguments and other types of controversy: in mathematics there is, when the dust has settled, a position that is correct and one that is not. At the start of the seminar series, Rourke believed that he and Rêgo had a correct proof. But when he realized they were mistaken, he himself pointed out the error.

That is the mathematical story, but it may not be the final word. Mathematical proof is similar to pregnancy: either you have one or you don't. But a research programme, a circle of ideas aimed towards a proof, is another matter. In a research programme, logical gaps in proofs are commonplace: that is how the work progresses. If the overall idea is sound, the gaps can usually be filled. There have been cases where proofs assumed to be defective because of gaps later turned out to be fundamentally sound: the work of Kurt Heegner on the class number one problem, or Louis de Branges on the Bieberbach conjecture, are examples. There have, conversely, been gaps that cannot be bridged: previous attempts on the Poincaré conjecture and all attempts so far at the Riemann hypothesis.

Rourke and Rêgo currently see their work as a programme rather than a proof. They hope to fill in the gap, or to get round it some other way. The smart money is of course that they will fail: it always is in such circumstances. Certainly, unless they succeed in patching up their proof, they will get no credit for having solved the problem — and rightly, because they have not. The ball is now firmly in Rêgo and Rourke's court. It is up to them to prove, if they can, that their programme can be carried through. If they cannot, Poincaré's conjecture will have notched up two more victims. □

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AIDS virus and scrapie protein genes

SIR—Haseltine and Patarca¹ have suggested regions of sequence similarity between the 3'-end of the polymerase gene of HIV (human immunodeficiency virus, type HTLV-III)² and the hamster gene for PrP 27–30, a protein associated with scrapie infectivity^{3,4}, but Braun and Gonda have since shown that the protein sequence alignments are unconvincing⁵. Compiling scores by the Needleman–Wunsch–Sellers algorithm (program ALIGN⁶) of sequence matches between the PrP gene and available lentiviral genes, including that of HTLV-III, we show here that the alignment of the DNA sequences is also without merit.

The regions of supposed nucleotide sequence similarity between PrP and HTLV-III are largely different from those for amino-acid sequences. Numerous gaps introduced into the DNA sequences for the purpose of alignment do not correspond to gaps in the protein sequence. Haseltine and Patarca's nucleic-acid alignment has four colinear regions of homology separated by regions of poor homology and different length. As in the case of protein alignment⁷, the number of gaps introduced lessens the alignment scores to the point where its significance is questionable. For example, alignment of the sequences of Fig. 1a of Haseltine and Patarca (PrP residues 207–631 and HTLV-III residues 4,632–5,123) with a low gap penalty of 2 (using the unitary scoring matrix of the PIR) and 100 randomizations, yields a score of 2.20 standard deviations (σ). PrP aligned with the analogous visna virus⁷ and equine infectious anaemia virus (EIAV)⁸ regions gives 0.87σ and -0.22σ , respectively. For comparison, alignments of the HTLV-III region with the analogous (and evolutionarily related) regions of the visna virus and EIAV genomes gives scores of 8.91 and 9.74 σ at the same break penalty.

Using the program FASTN⁹ to screen the GENBANK files, scores similar to, or in excess of those raised by the lentiviral-PrP alignments are frequent. For example, a section of the rat cytochrome P-450c gene¹⁰ (residues 4,723–5,203) matches HTLV-III (residues 4,637–5,123) with a score of 3.61 σ . Finally, regions other than the polymerase gene in lentiviral genomes give high ALIGN scores when compared with the PrP gene sequence. Thus, residues 5,277–5,751 of HTLV-III score 3.27 σ when aligned to PrP.

Haseltine and Patarca justify many of the gaps by the existence of small local (imperfect) repeats in the HTLV-III or PrP sequences. One of these 'repeats' is broken by the omission of the hexanucleotide GTGGAA following residue 261, an error which further extends a gap in the

HTLV-III sequence. Overall, the best patches of similarity occur in G–C rich parts of the amino-terminal halves of the genes.

Thus, any relationships between the amino-acid or nucleic-acid sequences of HTLV-III and PrP are probably fortuitous.

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1. Haseltine, W.A. & Patarca, R. *Nature* **323**, 115–116 (1986).
2. Ratner, L. *et al.* *Nature* **313**, 277–284 (1985).
3. Oesch, B. *et al.* *Cell* **40**, 735–746 (1985).
4. Basler, K. *et al.* *Cell* **46**, 417–428 (1986).
5. Braun, M.J. & Gonda, M.A. *Nature* **325**, 113–114 (1987).
6. Needleman, S.W. & Wunsch, C.D. *J. molec. Biol.* **48**, 443–453 (1970).
7. Sonigo, P. *et al.* *Cell* **42**, 369–382 (1985).
8. Stephens, R.M., Casey, J.W. & Rice, N.R. *Science* **231**, 589–594 (1986).
9. Lipman, D.J. & Pearson, W.R. *Science* **227**, 1435–1441 (1985).
10. Sogawa, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 5066–5070 (1984).

Interferon- β_2 living up to its name

SIR—The B-cell differentiation factor BSF-2 (or BCDF), whose complementary DNA was recently cloned from T cells by Hirano *et al.*¹, turned out to be identical to interferon- β_2 (IFN- β_2 , 26K), which we had cloned from human fibroblasts by means of its interferon activity^{2–4}. Billiau⁵, in pointing out this identity, questions the interferon-like function of the substance. We wish to recall the evidence which led us to classify this protein as an interferon and to consider the implications of these multiple activities.

Recombinant IFN- β_2 produced in CHO cells³ shows the following typical IFN activities. First, it inhibits vesicular stomatitis virus, Mengo virus, and herpes simplex virus-2 in human fibroblasts and amniotic cells^{3,6}. Second, it efficiently induces specific interferon-activated genes^{3,4}, such as (2'–5')-oligo (A) synthetase and induces their messenger RNAs in the presence of cycloheximide, showing the action to be direct⁷. Third, it has anti-mitogenic effects on fibroblasts⁸. Fourth, there is species specificity³. And finally, IFN- β_2 displays activity on mouse-human hybrid cells containing human chromosome 21, which carries the type I interferon receptor, and the activity is inhibited by antibodies to this receptor^{4,6,7}. Both the antiviral activity of IFN- β_2 and its ability to induce (2'–5')-oligo (A) synthetase are neutralized by several polyclonal and monoclonal antibodies to IFN- β_1 ^{3,7} (J. Vilcek and P.B. Sehgal, personal communication), but not by anti-

Quantum jump

SIR—Mention of our paper¹ in a recent News and Views article² leads me to emphasize that our experiments merely demonstrate a jumping time as short as about one second for the electron to complete the jump from the higher to the lower level. This is considerably shorter than the approximately 30 second dwell time in the upper electronic level, and the phenomenon therefore may legitimately be described as a 'jump'. It is not significantly shorter than our earlier example of a quantum jump³ although our later experiments have employed a different technique⁴ to observe the jump, namely a new continuous type of Stern–Gerlach effect.

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1. Negourney, W., Sandberg, J. & Dehmelt, H. *Phys. Rev. Lett.* **56**, 2797 (1986).
2. Maddox, J. *Nature* **323**, 577 (1986).
3. Van Dyck, R. Jr, Ekstrom, P. & Dehmelt, H. *Nature* **262**, 776 (1976).
4. Dehmelt, H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2291; 3074 (1986).

bodies to IFN- α or IFN- γ , hence its designation as a β -type interferon. Structurally, IFN- β_2 has conserved half of the amino-acid residues common to all type I interferons³. But by its gene structure and localization, IFN- β is only distantly related to the other interferons.

The finding of BSF-2 activity in an interferon molecule active on non-lymphoid cells, is not surprising in view of the known involvement of interferons in terminal differentiation³. With respect to B cells, IFN- α , β and γ have been shown to induce chronic lymphocytic leukaemia (CLL) cells to acquire plasmacytoid characters with increased cytoplasmic immunoglobulin⁸. The growth of some CLL B cells is strongly stimulated by IFN- α ⁸, a finding that may be related to the plasmacytoma growth stimulation mentioned by Billiau⁵. IFN- γ has been identified as a B-cell maturation lymphokine for normal murine B cells⁹ and, in the presence of interleukin-2, for human B cells¹⁰, producing strong increases in immunoglobulin secretion. Thus, interferons have B-cell differentiation effects.

IFN- β_2 , like IFN- γ , is produced by T cells, and both may exert lymphokine activity at concentrations lower than those giving antiviral effects¹¹. Indeed, IFN- γ induces HLA and anti-NK cell effects 100 times more efficiently than it inhibits viruses. IFN- β_2 has a specific activity 50–100 times lower than IFN- β_1 against vesicular stomatitis virus^{2,3} but its antimitogenic activity on fibroblasts is 10 times higher than IFN- β_1 (unpublished data), supporting the proposed role¹² of IFN- β_2 in fibroblast growth-control in response to

tumour necrosis factor or interleukin-1. It would, therefore, be not unlikely to find differentiation affects of IFN- β /BSF-2 on B cells at concentrations too low to produce antiviral activity on other cells. Contrary to Billiau's comment⁵, this does not mean that the interferon activity of IFB- β /BSF-2 is unimportant for its function: as it is as inducible as type I interferons by double-stranded RNA and viruses in many cells^{2,12}, IFN- β could provide an important link between the immediate tissue antiviral response and the more delayed antibody production in viral infections.

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- Hirano, T. *et al.* *Nature* **324**, 73-76 (1986).
- Weissenbach, J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 7152-7156 (1980).
- Zilberstein, A., Ruggieri, R., Korn, J.H. & Revel, M. *EMBO J.* **5**, 2529-2537 (1986).
- Revel, M. *et al.* in *The Biology of the Interferon System 1985* (eds Stewart, W.E. & Schellekens, H.) 207-216; 119-124 (Elsevier, Amsterdam, 1986).
- Billiau, A. *Nature* **324**, 415 (1986).
- Zilberstein, A. *et al.* in *The Biology of the Interferon System 1986* (eds Cantell, K. & Schellekens, H.) (Nijhoff, Boston, 1986).
- Zilberstein, A., Ruggieri, R. & Revel, M. in *The Interferon System* (eds Rossi, G.B. & Dianzani, F.) 73-83 (Raven, New York, 1985).
- Ostlund, L., Einhorn, S., Robert, K.-H., Juliusson, G. & Biberfeld, P. *Blood* **67**, 152-159 (1986).
- Sidman, C.L., Marshall, J.D., Shultz, L.D., Gray, P.W. & Johnson, H.M. *Nature* **309**, 801-804 (1984).
- Bich-Thuy, L.T. & Fauci, A.S. *J. clin. Invest.* **77**, 1173-1179 (1986).
- Wallach, D. *Cell. Immun.* **75**, 390-395 (1983).
- Kohase, M., Henriksen-DeStefano, D., May, L.T., Vitcek, J. & Sehgal, P.B. *Cell* **45**, 659-666 (1986).

Variations in chickens

SIR—Two recent reports in *Nature*^{1,2} suggest to us a possible mechanism for the generation of antibody diversity in chickens.

During immunoglobulin light-chain gene rearrangement, recombination occurs at conserved heptamer and nonamer nucleotide sequences which flank each germline variable (V) and joining (J) gene segment. Combinational joining among multiple non-identical V and J segments is a major source of antibody diversity in the mouse and human. In contrast, the principal light-chain locus of the chicken appears to contain only one J and only a single functional V segment, and fusion of this pair is the predominant (if not the only) rearrangement event observed. Other V-like elements exist upstream, but those sequenced to date are pseudogenes. Nevertheless, the chicken produces diverse light chains. Diversification appears to occur through transposition of sequences from pseudo-V segments into the rearranged V/J locus. The mechanism of this transposition is not known, but is thought to involve gene conversion³.

The finding^{1,2,4} that transcriptionally-competent heavy-chain genes can undergo secondary rearrangements in which one V

segment replaces another, with recombination occurring at a heptamer in the coding sequence of the first V segment, implies that the lymphocyte recombinase system can act on isolated heptamers contained in rearranged immunoglobulin genes. Upon examining the published sequences of the functional light chain V segment and three pseudo-V elements from the chicken, we find that each contains at least two heptamer elements within the coding region; these are located immediately upstream of the second and third complementarity-determining regions. It is unlikely that the pseudo-V loci undergo conventional rearrangements³. However, these internal heptamers, coupled with the putative heptamer-binding and endonucleolytic activities of the recombinant system, could serve as efficient sites for synapsis and strand exchange between the functional V segment and V-like pseudogenes, much as sequences flanking the mating-type loci of yeast are thought to do⁵. Resulting gene conversion events would tend to alter precisely those portions of the gene that determine antigen-specificity. Somatic diversification of the chicken light-chain genes might thus occur through a variation of the conventional heptamer-mediated recombinase pathway.

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- Reth, M., Gehrman, P., Petrac, E. & Wiese, P. *Nature* **322**, 840-842 (1986).
- Kleinfeld, R. *et al.* *Nature* **322**, 843-846 (1986).
- Reynaud, C.-A., Anquez, V., Dahan, A. & Weill, J.-C. *Cell* **40**, 283-291 (1985).
- Beck-Engeser, G.B., Jack, H.-M. & Wabl, M. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Strathern, J.N. *et al.* *Cell*, **31**, 183-192 (1982).

Functions of leaf fall

SIR—To add to those already put forward¹⁻³, may I suggest yet two more functions for leaf fall?

Some leaves may be shed because they cease to be economical in terms of their own and/or the whole plant's energy and nutrient budget. Such shedding would have the additional advantage of allowing a better distribution of light in the plant canopy, while minimizing competition for water and other nutrients from the soil.

It also seems possible that plants may initially produce many more leaves than they require, later shedding those that are in excess of the optimum number for the normal life cycle.

The above proposals gain support from the fact that in many of the economic legume and oil-seed crops, which can experience considerable leaf fall, reproductive structures can play an important role in the overall process of building seed yields, indicating that some leaves, at least, are

dispensable^{4,7}. The surplus foliage can account for at least half of the total plant foliage⁸. In fact considerable defoliation of such plants can be carried out, without a significant decrease in seed yields. Thus, in all plants where reproductive structures make a considerable contribution to the final seed yields, shedding of surplus foliage could be economical in the overall metabolic context of the plant and allow better distribution of light, water and nutrients.

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- Ford, B.J. *Nature* **323**, 763 (1986).
- Hardwick, R.C. *Nature* **324**, 517 (1986).
- Hendry, G. *Nature* **325**, 22, (1987).
- Crookston, R.K., O'toole, J. & Ozbun, J.L. *Crop Sci.* **14**, 708 (1974).
- Hardwick, R.C. *Br. Pl. Growth Regulator Group Monogr.* **9**, 61-74 (1983).
- Luthra, Y.P., Sheoran, I.S. & Singh, R. *Photosynthetica* **17**, 210 (1983).
- Willmer, C.M. & Johnston, W.R. *Planta* **130**, 33 (1976).
- Allen, E.J., Morgan, D.G. & Ridgman, W.J. *J. agric. Sci., Camb.* **77**, 339 (1971).

Body temperature of homoiothermic animals

SIR—I agree with Calder¹ and Dunitz and Benner² that Paul³ is wrong to suppose that the body temperature of many homoiothermic animals is 36 °C because that is approximately the temperature of minimum specific heat of water. Rather it may produce a paradox—homoiothermic animals live at the temperature of maximum thermal fluctuations. I also agree with Dunitz and Benner when they assert that biological water is not pure water but I disagree with them when they use the data of pure water to solve the paradox.

Indeed, water in biosystems must be thought of as adsorbed onto macromolecules, either in a localized or mobile form^{4,5}. Without additional hypotheses, this model explains the Na⁺/K⁺ ratio^{6,7}, the anaesthetic effect of inert gases⁸ and the anomalously high viscosity of water in the cell⁴; therefore, it must be considered as a plausible model for water in biosystems.

The observation that the specific heat of water in biosystems such as normal blood plasma and haematopoietic cells is lower (6% and 11%, respectively) than that of pure water, shows that in biological water some degrees of freedom can be excited with more difficulty than in pure water, thus sustaining the adsorbed water model.

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- Calder, W.A. *Nature* **324**, 418 (1986).
- Dunitz, J.D. & Benner, S.A. *Nature* **324**, 418 (1986).
- Paul, J. *Nature* **323**, 300 (1986).
- Cerofolini, G.F. & Cerofolini, M. *Specul. Sci. Technol.* **3**, 149 (1980).
- Cerofolini, G.F. *Adv. Colloid Interface Sci.* **19**, 103 (1983).
- Wiggins, P.M. *J. theor. Biol.* **32**, 131 (1971).
- Cerofolini, G.F. *Nuovo Cimento D2*, 763 (1983).
- Cerofolini, G.F. *Nuovo Cimento D2*, 1156 (1983).

Is seeing believing?

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Blindsight: A Case Study and Implications. By L. Weiskrantz. Clarendon: 1986. Pp.187. £19.50, \$29.95.

To See But Not To See: A Case Study of Visual Agnosia. By Glyn W. Humphreys and M. Jane Riddoch. Lawrence Erlbaum: 1987. Pp.124. Hbk £12.95, \$19.50; pbk £5.95, \$8.95.

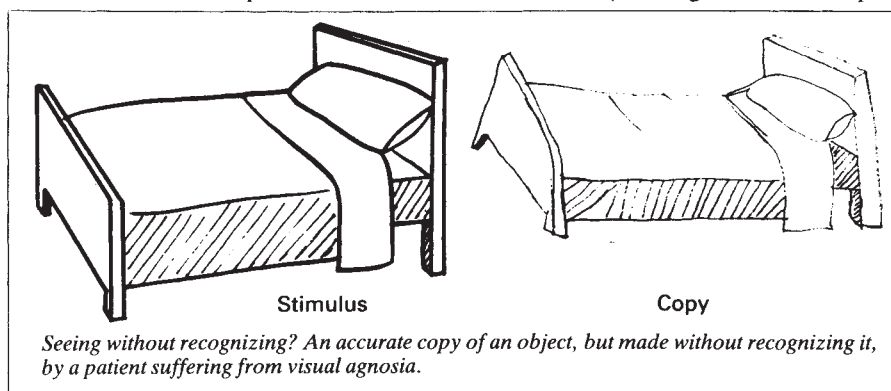
IF, IN good illumination (and wearing my glasses!) my eyes alight upon a common object, I have no difficulty in recognizing that there is, for example, a chair some two metres in front of me. This is the exercise of an everyday and seemingly trivial capacity, but one which has long frustrated our best endeavours to understand perception beyond the level of early visual processing. The problem of how the brain transforms the two-dimensional mosaic of retinal stimulation into the full richness of three-dimensional objects in space thus continues to intrigue neurophysiologists, experimental psychologists and workers in artificial intelligence (who would like their 'seeing machines' to recognize not only the numbers at the bottom of cheques, but also cabbages and kings).

To this list of interested parties we should add the neurologist and the neurosurgeon, who want to understand how damage to different areas of the brain can induce blindness in different parts of the visual field, or selectively impair the perception of form, colour, stereoacuity, orientation or location in space. In healthy condition, the visual system may operate too smoothly and seamlessly for us to grasp the *modus operandi* of its component parts. Thus, as with any other complex biological function, there is the strong possibility that pathology, by rudely tearing apart and isolating the elements of an integrated mechanism, will throw light upon the structure of the normal system.

To see how counter-intuitive that light may be, we need but turn to the case-reports presented by Weiskrantz and by Humphreys and Riddoch. The man, DB, that Weiskrantz has studied was born with an arteriovenous malformation at the pole of his right occipital lobe. The medical complications (migraine, severe pain and the possibility of life-threatening haemorrhage) were such that the malformation was removed surgically when DB was 33. Of necessity, the operation involved loss of much of the calcarine cortex on the medial surface of the right hemisphere. This area contains striate cortex that (by virtue of anatomical connectivity) is functionally characterized by an orderly retinotopic mapping of the visual world onto the surface of the brain.

Unsurprisingly, then, DB was left with

a visual field defect (a scotoma) that originally extended to the entire left half-field of both eyes, but later contracted to a scotoma of the lower left quadrants. In short, to perimetric testing and by all his own reports, DB was blind in these quadrants of the visual field. The puzzle is that he could reliably point to objects in this blind field, and could likewise discriminate between the horizontal or vertical orientation of a small stick exposed to the blind



field. This ability surprised the patient at least as much as it did the experimenters. DB continued to insist that the tasks he had been set were ridiculous and that he was simply 'guessing' the answers to questions that a (partially) blind man could not possibly answer correctly. Seeing without believing?

The man, HJA, investigated by Humphreys and Riddoch suffered a stroke at the age of 60. Blockage of the posterior cerebral artery provoked bilateral damage to the occipital lobes, including primary visual (striate) cortex. HJA, like DB, had a visual field defect; in this case, he was blind in the upper two halves of both the right and the left visual field. More crucially, however, HJA could not reliably identify by sight the faces of familiar people or the common objects in his environment even when he 'saw' them in his intact visual fields, a problem exacerbated by a concomitant loss of colour vision.

Is HJA, then, only 'partially sighted', even in his supposedly good lower visual fields? No. He has normal visual acuity (glasses-corrected); he can copy very accurately a drawing of St Paul's cathedral that hangs in his living room (although it takes him six hours to do so). The accom-

panying picture shows the copying performance of another patient, MS, with a deficit similar to that of HJA. We have no difficulty in seeing the copy for what it is, although the patient himself could not recognize that the object is a bed. Has HJA 'forgotten' what familiar objects and faces look like? No. He can verbally describe their visual characteristics from memory, and he can even draw quite accurately from memory such complex objects as a screw or a biplane. (Before the war he had trained to be a pilot.) Thus in some ways, but clearly not all, HJA is in a similar position to you and I when confronted with a novel object or new face. Seeing without recognizing?

Presented with such cases, a principled scepticism is in order. Thus attempts have been made to explain away blindsight on the grounds that some residual visual function is retained within the damaged striate area. (Blindsight is a limited phe-

nomenon, restricted to the detection of a target and relatively crude discriminations between simple stimuli.) Likewise, some of the experiments concerned have been criticized for leaving open the possibility that stray light reached intact portions of the visual field. The basic contrast between DB's retained ability to perform the tasks and his denial of 'seeing' the stimuli has led some to claim that the patient sets himself a higher 'confidence threshold' before being willing to say that he sees.

Much of Weiskrantz's book is accordingly taken up with a careful and detailed investigation of DB's visual abilities, extended over some dozen years and devoted to ruling out the obvious ways in which the interpretation of blindsight could be trivialized. To all save the direst sceptic, Weiskrantz's positive conclusions about the existence and nature of a 'second visual system', non-striate and "based on different cells of origin and different terminals, and with a different function" will seem alive and well. That the system has no (or very limited) access to conscious awareness reminds us (as does the current literature on the amnesias) that 'consciousness', anathema to the behaviourists, is not dead and will certainly not lie down. ▶

High-level visual agnosia, the deficit from which HJA suffers, has likewise been dismissed as an impossibility. Many neurologists have tried to explain away the performance of such patients by claiming that they suffered from a combination of low-level sensory problems aggravated by a generalized dementia. Once again, Humphreys and Riddoch's meticulous testing shows that such an explanation will not suffice. Apart from his problems with the visual recognition of objects, faces and written words, and with finding his way around his home town, HJA remains a highly capable and intelligent human being. He has excellent insight into his deficiency, and can describe his difficulties with remarkable cogency, although no words will do justice to the subjective feel of how his visual world has changed. Moreover, he has been able to devise sensible strategies for coping with the perceptual, intellectual and emotional consequences of his stroke.

Although these monographs provide appropriate literature-reviews and outline whatever background anatomical, physiological and psychological evidence is relevant to their theses, in both instances the primary data come from the study of a single individual. This provides striking confirmation of the methodological shift that cognitive neuropsychology has recently undergone. Group studies, in which a small range of observations is collected from a large sample of patients who are only superficially similar, either in terms of neuropathology or behavioural deficit, have proved an inadequate foundation on which to build models of human cognition. It is now widely recognized that single case-studies provide a more compelling, less ambiguous, database for inference to underlying mental representations and processes. Such investigations can furnish enough observations on the one patient to enable one to home in on the precise information-processing mechanisms involved in the pattern of impaired and preserved performance. The research strategy is well illustrated in the sequence of experiments whereby Humphreys and Riddoch isolated HJA's primary deficit: a disorder in "binding together" the local parts of objects simultaneously at different spatial locations".

Of course, the deficits of one patient cannot reveal the overall structure of the visual system. But from a series of cases, each with a different selective disorder, we may reasonably hope to discover the functional architecture of visual cognition. It is in this sense that *Blindsight* and *To See But Not To See* are important steps towards the eventual maturity of cognitive neuropsychology as a natural science. □

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The comparative advantages

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Calcium in Muscle Activation: A Comparative Approach. By Johann Caspar Rüegg. Springer-Verlag: 1986. Pp.300. DM 198.

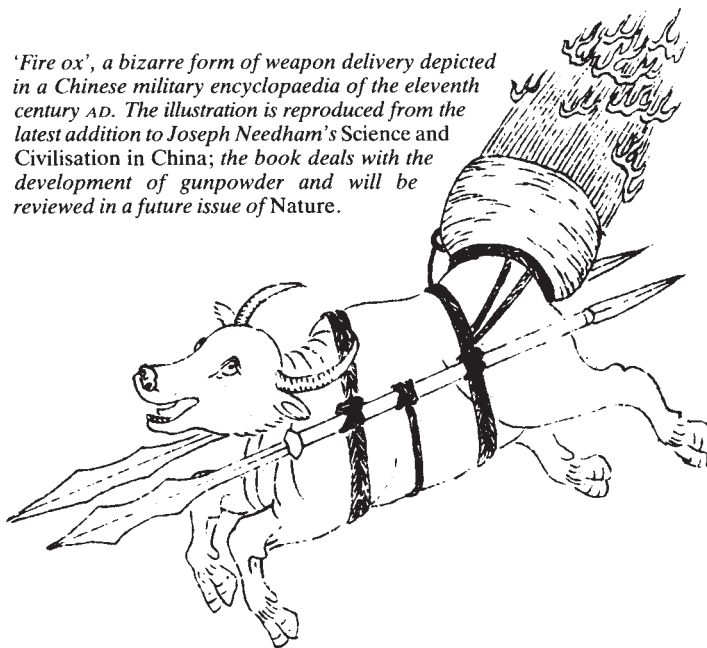
As more problems of biology approach a molecular level, the chemical and physical sciences make an ever-increasing contribution to the advances in our understanding. Comparative physiology might seem a backwater, followed by those whose originality limits them to choose a new organism rather than a new experiment upon which to work. Rüegg sets out to dispel this viewpoint, quoting in the Introduction his predecessor Krogh, who advised experimentalists to search for the most specialized organisms or tissues in order to explore mechanism.

Throughout the book, many examples are given where new experiments were made possible by the choice of tissue rather than an advance in technology *per se*. Take Ca^{2+} itself—the central theme of this work. Muscle physiologists were aware of the importance of this ion as a messenger long before its acceptance as a trigger in a host of other cellular functions. With a head start of nearly a century, the molecular bases of activation mechanisms are better understood in muscle (but by no means solved) than in any other system. Indeed, calmodulin was known as troponin-C-like protein just a decade ago, before its explosive rise to fame in metabolism as a whole.

A second strength of the comparative physiological approach is as an aid to understanding the generation of diversity through evolution. Already the euphoria of some protein engineers has been dampened by the realization that nature has had more than 10^9 years to experiment with amino-acid substitutions. Its successes are here today, but are in danger of being forgotten as classical zoology and botany are squeezed out of the syllabus. Muscle from different organisms shows over a 10,000-fold range in shortening speeds. This is accomplished by diversity in the rate and mode of stimulation, the diffusion distance of the Ca^{2+} message, and the properties of the Ca^{2+} -receptor proteins, Ca^{2+} pumps and contractile machinery. Input is therefore required from plant and animal anatomy, physiology and biochemistry to gain a full understanding. Yes, plants do have muscles of a sort and knowledge of their anatomy has provided an elegant way to demonstrate the velocity of shortening is, in part, controlled by the particular myosin isoenzymes present in a muscle. The giant algal cell *Nitella* contains strands of actin running along its length which provide an *in vitro* substrate along which latex beads, covered with myosin, crawl with a velocity dependent on the muscle source. But much of the diversity of muscles is a reflection of their microanatomy which would be missed by the study of isolated proteins alone.

There are few people of Rüegg's experience to document these comparative aspects of activation of muscle contraction so thoroughly, and who could blend the disciplines involved so effectively and do justice to both the history of the subject and the latest discoveries. The individual

'Fire ox', a bizarre form of weapon delivery depicted in a Chinese military encyclopaedia of the eleventh century AD. The illustration is reproduced from the latest addition to Joseph Needham's *Science and Civilisation in China*; the book deals with the development of gunpowder and will be reviewed in a future issue of *Nature*.



chapters are more or less self-contained, and cover excitation-contraction coupling mechanisms in a variety of vertebrate and invertebrate muscle types. There is some repetition, but this is no bad thing as most readers are likely to refer to a section at a time. The writing is lucid and most of the figures have been redrawn from the literature in a clear, consistent format. Controversies are generally dealt with by presenting both sides of the argument, without forcing premature conclusions.

Nevertheless, the various topics are held together with the author's own views,

which the reader may or may not share. Thus, in a comparison of actin- and myosin-linked regulation, Rüegg considers that the former is more efficient because there is "a built-in guarantee that crossbridges do not hydrolyse ATP unless they are attached to actin", yet it is known that skeletal myosin alone hydrolyses ATP *in vitro* at a suspiciously high rate compared with a relaxed muscle. Some additional way of modulating the myosin ATPase may therefore be implicated. □

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All about leeches

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Leech Biology and Behaviour. By Roy T. Sawyer. Oxford University Press:1986. Pp. 1,065 in three volumes. Vol. I £47.50, \$85; Vol. II £42.50, \$85; Vol. III £25, \$50.

PERHAPS the question that almost all invertebrate zoologists would ask about books of the type written by Sawyer is "How do they compare to Libbie Hyman's volumes on the invertebrates?". The short answer is in many respects very well, but in other respects poorly.

The three volumes — *Anatomy, Physiology, and Behaviour*; *Feeding Biology, Ecology, and Systematics*; and *Bibliography* — cover almost all aspects of the subject matter implied by their titles, but the emphasis is upon the use of leeches in modern medicine and particularly the numerous chemicals of pharmacological use produced by leeches. Each topic is covered extensively and as a source of information the books are excellent. If, however, the reader is looking for an holistic approach fitting leeches into a more general framework, there are disappointments. This particularly applies to the ecological section, which has little interpretative content and fails to establish substantive links with mainline ecology.

Thus, like Hyman, if information is required Sawyer will generally provide it. But, unlike Hyman, there is no balanced analysis of possible phylogenetic development. Only an enthusiastic leech researcher would seriously contemplate reading these books from start to finish, but by beginning with a very personal and idiosyncratic account of the phylogenetic affinities of the leeches Sawyer will probably deter all but the most dedicated. My greatest fear is that these introductory chapters may well not only put off the casual reader but will potentially misinform or bias the unwary. For example, Sawyer suggests that certain traits reflect a monophyletic link between the Clitellata and the Uniramea, with the Polychaeta

having a separate and distinct origin. Alternative views are largely ignored or barely mentioned. The dogmatic adherence to this phylogenetic perspective pervades almost every chapter and detracts from an otherwise excellent work of scholarship. Similarly, the inclusion of the Acanthobdellida and Branchiobdella within the leeches (Hirudinea) deserves more discussion, and alternative phylogenetic pathways should have at least been mentioned.

The sections on systematics, evolution and zoogeography will be extremely useful to leech researchers throughout the world, although it is a pity that Sawyer does not fully explain the taxonomic changes he has instituted. Most leech researchers are well acquainted with the publications of Soos and Richardson on leech genera of the world (published in *Acta Zoologica Academiae Scientiarum Hungaricae*). However, many of the genera, especially in the Hirudinidae, so carefully defined by these workers have been eliminated largely without explanation. Inclusion of this information would have undoubtedly increased the value of Sawyer's books.

The separation of the chapter on behaviour in Vol. I from those on feeding biology and ecology in Vol. II is difficult to understand, as is the separation into different volumes of phylogeny and systematics. With a common index for Vols I and II and the bibliography in Vol. III, most readers will be required to juggle two if not three volumes coincidentally. Undoubtedly space was saved as a result, but at the cost of seriously inconveniencing the user.

The books are well written and illustrated, and despite (or perhaps because of) Sawyer's personal biases on phylogeny I found them enjoyable and stimulating. The author must be congratulated on a monumental achievement, which will be invaluable to dedicated leech researchers and a must for their laboratories and every university library. □

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Malevolent moulds on record

Maurice O. Moss

Fusarium Species: Their Biology and Toxicology. By Abraham Z. Joffe. Wiley: 1986. Pp.588. \$74.95, £71.75.

IT WAS just six years ago that a speech made in West Berlin by US Secretary of State Alexander Haig threw the mould genus *Fusarium* into the spotlight of the news media. The speech linked the phenomenon of 'yellow rain' in South-East Asia with the production and use of the toxic mould metabolites known as trichothecenes as agents of chemical warfare. Although 'yellow rain' is probably the pollen-laden faeces of bees, and the truth about the use of chemical weapons in South-East Asia is distorted by the murky clouds of superpower politics, the importance of *Fusarium* in natural cases of poisoning of man and domesticated animals is well recognized.

Severe outbreaks of illness associated with the consumption of cereals contaminated with species of *Fusarium* and other moulds occurred in parts of the Soviet Union during the first half of this century. This illness, known as alimentary toxic aleukia (ATA), is associated with a complex pattern of debilitating disorders which starts with inflammation of the mouth and throat and is followed by damage to the mucosal membrane systems of the intestine and associated vomiting and diarrhoea. With increased consumption of toxic cereals, damage to the bone marrow occurs followed by a complex anaemia. The trichothecenes are now considered to be responsible for ATA. Like several other mycotoxins they are immunosuppressive, so the body's defences against bacterial and viral infections are reduced; indeed, many deaths during outbreaks of ATA were put down to pulmonary and other infections.

During a major Russian outbreak of ATA in the 1940s, Abraham Joffe was Director of the Mycology Laboratory of the Institute of Epidemiology and Microbiology in Orenburg. He was closely involved in investigations of the aetiology of the disease and continued to specialize in the study of toxigenic fusaria as head of the Laboratory of Mycology and Mycotoxicology at the Hebrew University of Jerusalem. *Fusarium* is only one of a number of mould genera which are known to produce toxic metabolites (zearalenone, butenolide and moniliformin, for example, as well as the trichothecenes) and there is now a considerable body of literature on the mycotoxigenic fungi, mycotoxins and mycotoxicoses. Professor Joffe has made continuous and notable contribu-

tions to this literature, and the book under review represents the accumulation of his experience with *Fusarium*.

The book begins with a brief historical background account, which is followed by chapters on the toxigenic fusaria and their principal toxins; included here are tables of yields of toxins produced in the laboratory, concentrations found during instances of natural occurrence, and values, taken from the literature, of their toxicity in terms of LD₅₀. Next come discussions of antibacterial, antiprotozoal and insecticidal activity (but not of antifungal activity, despite the existence of several reports on the use of yeasts in the bioassay of trichothecenes) and of the phytotoxicity of *Fusarium* species. The latter chapter is dominated by the author's own studies on correlation between the rabbit skin test and phytotoxicity — one hopes that such tests will soon be made obsolete by developments in immunological assays, biological assays using microorganisms and cell cultures, and modern physico-chemical analytical methods.

The remaining chapters are devoted to human fusariotoxicooses, the role of *Fusarium* in human mycoses, fusariotoxicooses of laboratory and domestic animals,

and the difficult issues of the taxonomy and identification of toxigenic isolates (keys and detailed descriptions and synonymies are provided). Professor Joffe finishes where I began — with a comment on the question of 'yellow rain'.

The contents are essentially a compilation of primary data, and this is not an easy book to read. It gives the reader the feeling that he or she is looking over the author's shoulder directly into his laboratory records. Compounding the problem is the large number of references interspersed throughout the text. The resulting bibliography fills 86 pages and spans the period from 1890 to 1984.

Despite the difficulty of assimilating all of the information presented in this book, no one will be left in any doubt about the toxigenic nature of many isolates of *Fusarium*. A bonus for those interested in biological variability is that it is unusual to find comparative data on such a large collection of isolates, and the book provides useful insight into the range of toxigenic capability amongst isolates of the same species. □

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How to play with particles

Robert J. Hollebeek

Introduction to Experimental Particle Physics. By Richard C. Fernow. Cambridge University Press: 1986. Pp. 421. £32.50, \$44.50.

Statistics for Nuclear and Particle Physicists. By Louis Lyons. Cambridge University Press: 1986. Pp. 226. £25, \$44.50.

Detectors for Particle Radiation. By Konrad Kleinknecht. Cambridge University Press: 1986. Pp. 206. £25, \$44.50.

EXPERIMENTAL elementary particle physics has changed so rapidly during the past decade that few of its experts have attempted to describe the current state of research in anything but conference proceedings and contributions to technical journals. The problem is that having spent years attempting to write a comprehensive review of the subject, an author may find himself in possession of an excellent but out-dated volume. For the student, however, this situation makes it quite difficult to become an expert except by working directly on a high-energy physics experiment and reading extensively in current journals. Even this is sometimes not enough, since many experiments are now done by several hundred physicists and take many years to complete. As a result, a student may be quite knowledgeable

in a specific area, and yet know little about many of the other techniques being used on his own experiment. It is a brave author indeed who embarks on publishing a text in such a field, and the rest of us should be suitably thankful that some still do.

Richard Fernow's *Introduction to Experimental Particle Physics* is an excellent step towards bridging the gap between the enormous amount of primary literature and the beginning graduate student or interested scientist. The emphasis here is not on theories but on techniques. If you wish to learn about quarks, unified field theories, strings, supersymmetry and weak gauge theories, you should look elsewhere; but if you want to learn about the many techniques used to design a detector capable of probing inside the smallest distances known to man, this is a good place to start.

The introductory chapters cover the various interactions of charged and neutral hadrons and leptons in matter in sufficient detail for a graduate-level course. The author proceeds from this background to discuss the types of detectors which make use of these physical processes, the electronics systems used to read them, and the machines and beams used to do the physics. In many cases, comparisons are made between different techniques and suggestions given to the experimenter on how to optimize a detector for a particular application. There are a large number of references, many of which cite major review articles

or important journal papers, and each chapter concludes with a small number of exercises.

Also of interest to experimental particle physicists, as well as anyone else who has to use statistical techniques, is Louis Lyons's *Statistics for Nuclear and Particle Physicists*. This is probably not the first book on statistics which a student or researcher should read, because many results from statistical analysis are stated or provided in a non-rigorous way. It is instead, as the author states, the result of "a course given by a non-statistician to non-statisticians". While the title states that the book is intended "for nuclear and particle physicists", this is probably too restrictive; many examples are drawn from particle physics, but they are simple enough to be understood by the non-expert.

The book's true value is that it contains the accumulated experience and insight of someone who has learned to use statistics to solve problems encountered by scientists in general. Many simple examples are given in which the author emphasizes the connections between various statistical techniques and the most frequent errors made in applying them. This, then, is an ideal book for the person who knows about statistical techniques but wants to learn how and why to apply them. In considering the origin of the well-known $N-1$ term in the sample variance, for example, Lyons points out the advantages and disadvantages of using several other choices of the form $N + k$ (one instance is that using $N + 1$ yields a biased estimator, which has a smaller variance about the true variance). When correlated variables are introduced, Lyons starts with the simple example of Abram and Lot at the cross-roads. In Lyons's words, "The reader interested in further details should consult Genesis 13:9". A challenging set of problems accompanies each chapter, many of which expand on concepts discussed in the text.

Detectors for Particle Radiation by Konrad Kleinknecht is the most detailed of the books under review, and consequently will become dated most rapidly. Several of the areas covered are already in need of changes and additions; for example, semiconductor detectors are described but no mention is made of advances in the past few years in CCD and silicon strip detectors, and the account both of RICH counters and compensation in hadron calorimeters needs to be updated. The discussion of the physical processes which are used in different types of detectors is briefer than Fernow's, and probably less useful for graduate students, but researchers using wire chambers or scintillators will find here an outstanding review of their speciality. □

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Seasonal temperatures in Britain during the past 22,000 years, reconstructed using beetle remains

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Seasonal and mean annual temperatures can be reconstructed from radiocarbon-dated beetle remains to yield a synthesis of the palaeoclimatic history of Britain over the last 22,000 years. The reconstructions agree well with independent evidence from periglacial features and provide a detailed picture of the glacial to interglacial transition 14,500–9,000 years ago.

IN this paper we present a detailed reconstruction of temperature changes for both summer and winter in the British Isles, based upon radiocarbon-dated occurrences of fossil *Coleoptera* (beetles) covering the period between the last glacial maximum and the present day. Britain is a particularly suitable area for such study since it lies at the south-west edge of the former European ice-sheets, and adjacent to the region of the North Atlantic which was traversed by migrations of the front between polar and sub-polar water masses¹. There is also a 30-year history of studies of fossil *Coleoptera* and their climatic interpretation, which provides data which are unmatched elsewhere in the world.

Principles of palaeoclimatic reconstruction

The palaeoclimatic reconstructions described below were made by a new technique, the mutual climatic range method, which is derived from early work on pollen by Iversen² and Grichuk³. The method has been fully described elsewhere⁴ but the principle is extremely simple. The basic assumption is that, if the present day climatic tolerance range of a beetle species is known, then fossil occurrences of that species imply a palaeoclimate which was within the same tolerance range. If several species occur together as fossils, then the palaeoclimate of the time when they were living must lie within the mutual intersection of their tolerance ranges. In general the intersection (or Mutual Climatic Range) will be smaller and the palaeoclimate deduced more precisely, the larger the number of co-existing species. Figure 1a shows two tolerance ranges and their mutual climatic range (MCR).

Coleoptera are especially suited to this technique because they are a varied group in which many species show fairly well defined tolerance ranges, they are well preserved as fossils, and almost all Pleistocene occurrences can be identified as belonging to living species. It has been shown⁵ that on a hemispheric scale the distribution of individual species reflects the thermal climate, especially the warmth of the summer and the degree of continentality or temperature contrast between summer and winter, and that the species compositions of fossil beetle assemblages respond rapidly to climatic change. This last point has been confirmed by Hengeveld⁶ who has shown that the relative abundances of species making up the present-day fauna of the Netherlands has changed in response to climate on a decadal timescale since 1890.

For this study, we have defined the climatic tolerance ranges of 350 beetle species which occur as Pleistocene fossils. Only carnivorous species from the families Carabidae, Dytiscidae, Gyrinidae, Halplidae and Hydrophilidae and dung-beetles (Scarabaeidae) were included. We have avoided using phytophagous beetles, whose distributions might reflect that of their host plants. A map of each species' present distribution

was compiled from information in the entomological literature and museum specimens. Each map was then transformed into a diagram of the species' distribution in terms of climatic variables, by superimposing the geographical range onto a base map of 495 climate stations scattered across the Palaearctic region from Greenland to Japan. Principal components analysis of the mean monthly temperatures from these stations shows that over 96% of the variance in thermal climate of the Palaearctic is described by two groups of variables (principal components) which can be interpreted as (1) the warmth of the summer and (2) the temperature range between warmest and coldest months. As the mean temperature of the warmest month (TMAX) is almost perfectly correlated with the first component, and the temperature range (TRANGE) is similarly correlated with the second component, these two variables were used directly to express the warmth of the climate and its degree of continentality.

The 495 stations were plotted on a diagram of TMAX against TRANGE. For each species, the stations which lie within its geographical range were distinguished, from which can be determined the species climatic range (SCR). The validity of this procedure was demonstrated when disjunct geographical distributions became, on replottting, coherent climatic ranges.

To reconstruct the MCR of an assemblage of coexisting species, the SCRs of all members of the assemblage are superimposed, as in Fig. 1a. From the extent of the MCR on such a diagram, we may read off the ranges of TMAX and TRANGE within which the palaeoclimate should fall. The range of mean temperatures of the coldest month (TMIN) may also be read from the diagram. Of course, we cannot be sure exactly what value the palaeoclimate had, only that it lay somewhere within the MCR.

A great advantage of the MCR is that it is possible to test its accuracy by reconstructing the climate from living assemblages of *Coleoptera* and checking the results against nearby climate stations. We have applied the method to lists of the species found in 15 restricted localities (such as nature reserves and small islands) in Iceland, Europe and Siberia, plus one site in Labrador. In Fig. 1b the reconstructed ranges of possible values for TMAX and TMIN are compared with the actual climates of each locality. There is excellent agreement. Overall, the MCR method provides an accurate indication of the range of values within which the climate of an assemblage should fall.

We now turn our attention to the problem of estimating the 'most probable' value of the palaeoclimate within the MCR. As a first approximation we may take the median values of the possible ranges for TMAX and TMIN. However, Fig. 1b shows that these tend to provide a systematic deviation from the 45° line with the reconstructed median lying above the true value of TMAX in all but the most temperate climates. With TMIN,

the median overestimates the true value in colder climates but underestimates it for localities with mild winters. These systematic deviations can be corrected using regression equations of the present-day temperatures at various localities against the median reconstructed temperatures based on the *Coleoptera* from the same localities. To derive these regressions we used two independent sets of data comprising, firstly, the fifteen localities shown in Fig. 1b and, secondly, faunal lists from 31 geographical provinces of Scandinavia and Denmark reported by Hansen *et al.*⁷. The latter must be used with caution because the lists comprise species collected over wide areas with, in some cases, internal variations in climate, and may include species whose climatic ranges do not overlap. Nevertheless, when separate regressions for TMAX and TMIN were derived from each data set and tested on the other, satisfactory results were obtained, so the two data sets were combined to produce the correction equations,

$$\text{TMAX}(\text{corrected}) = 1.066 \text{TMAX}(\text{median}) + 0.0142 \text{NSPEC} - 2.96 \quad (1)$$

$$(r = 0.94; s = 0.83^\circ\text{C})$$

$$\text{TMIN}(\text{corrected}) = 1.416 \text{TMIN}(\text{median}) + 1.904 \quad (2)$$

$$(r = 0.94; s = 2.42^\circ\text{C})$$

where temperatures are in $^\circ\text{C}$ and NSPEC is the number of species used in the reconstruction. Full details of this calibration procedure will be published elsewhere. 'Corrected' values obtained using equations (1) and (2) provide unbiased estimates of the most probable palaeoclimate within the MCR, with a precision of $\sim \pm 2^\circ\text{C}$ for TMAX and $\sim \pm 5^\circ\text{C}$ for TMIN.

Results for British Isles, 22,000 yr BP to now

The MCR method has been applied to 57 coleopteran faunas from 26 sites in England, Ireland and Southern Scotland. Site locations are shown in Fig. 2a and details are given in Table 1. Forty of the faunas are directly associated with a radiocarbon date on material from the same stratum. At only two sites, Glanllynau⁹ and St Bees¹⁰, was there a sufficiently long and detailed stratigraphic section to allow dating by interpolation between radiocarbon-dated horizons. Between them, these two sites provided 6 directly dated faunas and 17 faunas dated by interpolation between 14,500 and 11,500 yr BP.

The results are shown in Fig. 2b and 2c. Figure 2b presents values of TMAX and TMIN in the form of vertical bars indicating the MCR of each fauna. The position of each bar on the time axis of the diagram is given by its radiocarbon or interpolated date. Dating uncertainties are not shown. One-sigma errors vary from ± 45 to ± 325 yr, averaging ± 120 yr ($s = 62$).

Figure 2b shows several periods of very rapid climatic change, especially the dramatic warmings at $\sim 13,000$ yr BP and $\sim 10,000$ yr BP and a slower cooling from $\sim 12,500$ to $10,500$ yr BP. These changes occur progressively over a series of points on the graph and their timing and rapidity are therefore well established. However, there is also weaker evidence for minor excursions of the climate away from the major trends. The question of whether these minor excursions are real features or mere artefacts of the uncertainty in the data depends critically upon the accuracy and precision of individual radiocarbon dates.

Figure 2c was prepared as an attempt to overcome some of the dating uncertainties surrounding individual points on Fig. 2b, by presenting time-averaged temperature estimates calculated at 50-year intervals. For each 50-year point, all the temperature estimates whose dates overlapped the point at the 3σ level were identified. The weighted mean of these temperatures was then calculated, using weightings determined as follows. The separation of each dated temperature estimate from the time point under consideration was expressed as a multiple, X , of the 1σ standard error of the date. The weighting factor for

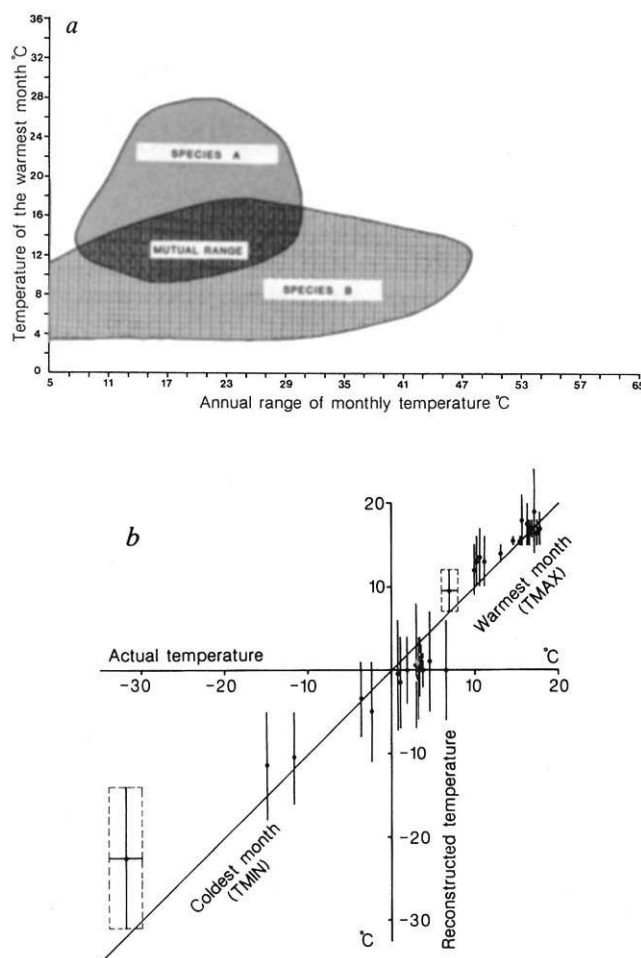


Fig. 1 a, Tolerance ranges and mutual climatic range (MCR) of two species of coleoptera. The tolerance ranges were determined by the method described in the text. b, Test of the MCR method on assemblages of species found living today at fifteen localities in Europe, Iceland and Siberia. The reconstructed temperatures of the warmest and coldest months of the year (TMAX and TMIN) are shown on the vertical axis. Points representing TMIN lie below and to the left of the upper boxed point. Vertical bars are not conventional error bars. They indicate the MCR, that is the predicted range of temperature within which all of the species at each locality could co-exist. Dots show the median value of the MCR at each locality. The horizontal axis shows the actual mean temperatures at climate stations close to each locality. Boxed points represent the coldest locality studied, Chaun Bay in north-west Siberia, for which actual temperature data were interpolated from a climatological atlas. The MCR method gives an accurate prediction of the climate for all points whose vertical bar overlaps the 45° diagonal line.

that temperature estimate was calculated as the ordinate of the normal curve at distance $X\sigma$ from the mean. Thus, each individual data point in Fig. 2b has been 'spread' over a time range determined by the precision of its date and combined with other points similarly 'spread'. This procedure is not totally satisfactory in that its effects depend somewhat upon the spacing of dates in time as well as their uncertainties, but it is an objective way of producing a single line through the scatter of data. The influence of single anomalous points is reduced and the timing of major changes reflects the uncertainties of all of the individual dates.

The bold lines shown in Fig. 2c represent the best estimates of palaeotemperature obtained by applying equations (1) and (2) to the median values of the MCR. The shaded lines show the upper and lower bounds of the MCR itself. Thus, the most

Table 1 Locations and dates of Coleopteran faunas

Site*	Number on Fig. 2	UK National Grid Reference	Date§	Lab. No.	NSPEC	Publication reference
Lea Valley, N. London	1	TQ357936	21,580 ± 480	BIRM-238	5	33
Barnwell Station, Cambridge	2	TL462573	19,500 ± 650	Q-590	12	34
Dimlington, E. Yorks	3	TA391271	18,370 ± 334	I-3372	4	35
				BIRM-108		
Glanllynau, Gwynedd	4	SH455373	14,470 ± 300	BIRM-212	6	9
Glanllynau, Gwynedd	4	SH455373	14,110 ± 380	Interpolated	6	9
Glanllynau, Gwynedd	4	SH455373	13,840 ± 380	Interpolated	3	9
Glanllynau, Gwynedd	4	SH455373	13,570 ± 380	Interpolated	2	9
Stafford, Staffs.	5	SJ927233	13,490 ± 375	BIRM-150	5	36
Colnbrook, W. London	6	TQ026776	13,405 ± 170	Q-2021	6	37
Glanllynau, Gwynedd	4	SH455373	13,300 ± 380	Interpolated	2	9
Glanllynau, Gwynedd	4	SH455373	13,165 ± 380	Interpolated	4	9
Glanllynau, Gwynedd	4	SH455373	13,030 ± 380	Interpolated	6	9
Roberthill, Dumfriesshire	7	NY110797	12,940 ± 250	Q-643	7	38
Glanllynau, Gwynedd	4	SH455373	12,925 ± 380	Interpolated	10	9
Glanllynau, Gwynedd	4	SH455373	12,860 ± 380	Interpolated	8	9
Glanllynau, Gwynedd	4	SH455373	12,725 ± 380	Interpolated	10	9
Glanllynau, Gwynedd	4	SH455373	12,655 ± 380	Interpolated	21	9
Glanllynau, Gwynedd	4	SH455373	12,590 ± 380	Interpolated	50	9
St. Bees, Cumbria	8	NX965114	12,560 ± 170	BIRM-378	43	10
Glanllynau, Gwynedd	4	SH455373	12,556 ± 230	BIRM-276	59	9
St. Bees, Cumbria	8	NX965114	12,405 ± 210	Interpolated	25	10
St. Bees, Cumbria	8	NX965114	12,255 ± 210	Interpolated	28	10
West Bromwich, W. Midlands	9	c.SP020940‡	12,165 ± 160	IGS-C14/11	4	39
				St. 3059		
Shortalstown, Co. Wexford	10	T303114†	12,165 ± 180	I-4963	20	40
St. Bees, Cumbria	8	NX965114	12,100 ± 210	Interpolated	21	10
St. Bees, Cumbria	8	NX965114	11,945 ± 210	Interpolated	21	10
St. Bees, Cumbria	8	NX965114	11,790 ± 210	Interpolated	18	10
Lea Marston, Warwicks.	11	SP210940	11,700 ± 200	BIRM-208	21	41
Bigholm Burn, Dumfriesshire	12	NY316812	11,700 ± 180	Q-694	25	38
St. Bees, Cumbria	8	NX965114	11,635 ± 210	Interpolated	14	10
St. Bees, Cumbria	8	NX965114	11,500 ± 120	BIRM-647	24	10
Wilden, Worcs.	13	SO824730	11,420 ± 480	BIRM-1020	4	42
St. Bees, Cumbria	8	NX965114	11,300 ± 220	BIRM-648	19	10
Northmoor, Oxon.	14	SP419041	11,250 ± 100	BIRM-105	9	43
West Drayton, W. London	15	TQ053792	11,230 ± 120	Q-2030	6	37
Redkirk Point, Dumfriesshire	16	NY301652	11,205 ± 177	BIRM-41	28	38
St. Bees, Cumbria	8	NX965114	11,180 ± 120	BIRM-649	31	10
Redkirk Point, Dumfriesshire	16	NY301652	10,898 ± 127	BIRM-40	24	38
Farmoor, Oxon.	17	SP452059	10,600 ± 250	BIRM-590	22	43
Brimfield, Herefordshire	18	SO389681	10,625 ± 282	BIRM-404	21	44
Drumurcher, Co. Monaghan	19	H521172†	10,515 ± 195	BIRM-239	20	45
St. Bees, Cumbria	8	NX965114	10,430 ± 165	BIRM-1144	8	10
Croydon, Surrey	20	TQ297656	10,130 ± 120	BIRM-101	5	46
West Bromwich, W. Midlands	9	c.SP020940‡	9,970 ± 100	IGS-C14/12	7	39
				St. 3060		
Wilden, Worcs.	13	SO824730	9,930 ± 220	BIRM-905	10	42
Wilden, Worcs.	13	SO824730	9,860 ± 240	BIRM-907	2	42
Brighouse Bay, Kirkudbrightshire	21	NX634452	9,640 ± 180	Q-398	15	38
West Bromwich, W. Midlands	9	c.SP020940‡	9,540 ± 100	IGS C14/82	3	39
				St. 3693		
Lea Marston, Warwickshire	11	SP210940	9,450 ± 200	BIRM-215	38	47
				BIRM-310,311,312		
				BIRM-329		
West Bromwich, W. Midlands	9	c.SP020940‡	9,080 ± 455	IGS-C14/83	5	39
				St. 3688		
Alcester, Warwickshire	22	SP099577	8,480 ± 140	BIRM-709	4	48
Alcester, Warwickshire	22	SP099577	8,320 ± 100	BIRM-860	5	48
Shustoke, Warwickshire	23	SP227908	4,830 ± 100	NPL-39	19	49
Wilsford, Normanton Gorse, Wilts.	24	c.SU1280‡	3,330 ± 90	NPL-74	25	50
Porthmear, Cornwall	25	SW848715	3,024 ± 126	BIRM-197	4	51
Fisherwick, Staffs.	26	c.SK175098‡	2,130 ± 100	BIRM-614	19	52
Shustoke, Warwickshire	23	SP227908	410 ± 90	NPL-62	25	49

* Sites are given in order of date and can be identified on Fig. 2a by number and on Fig. 2b by counting bars from the left.

† Irish Republic Grid Reference on 1:68360 maps.

‡ Approximate Grid Reference (not given in relevant publication).

§ Radiocarbon Years BP with 2σ counting error.

|| Number of species in MCR method.

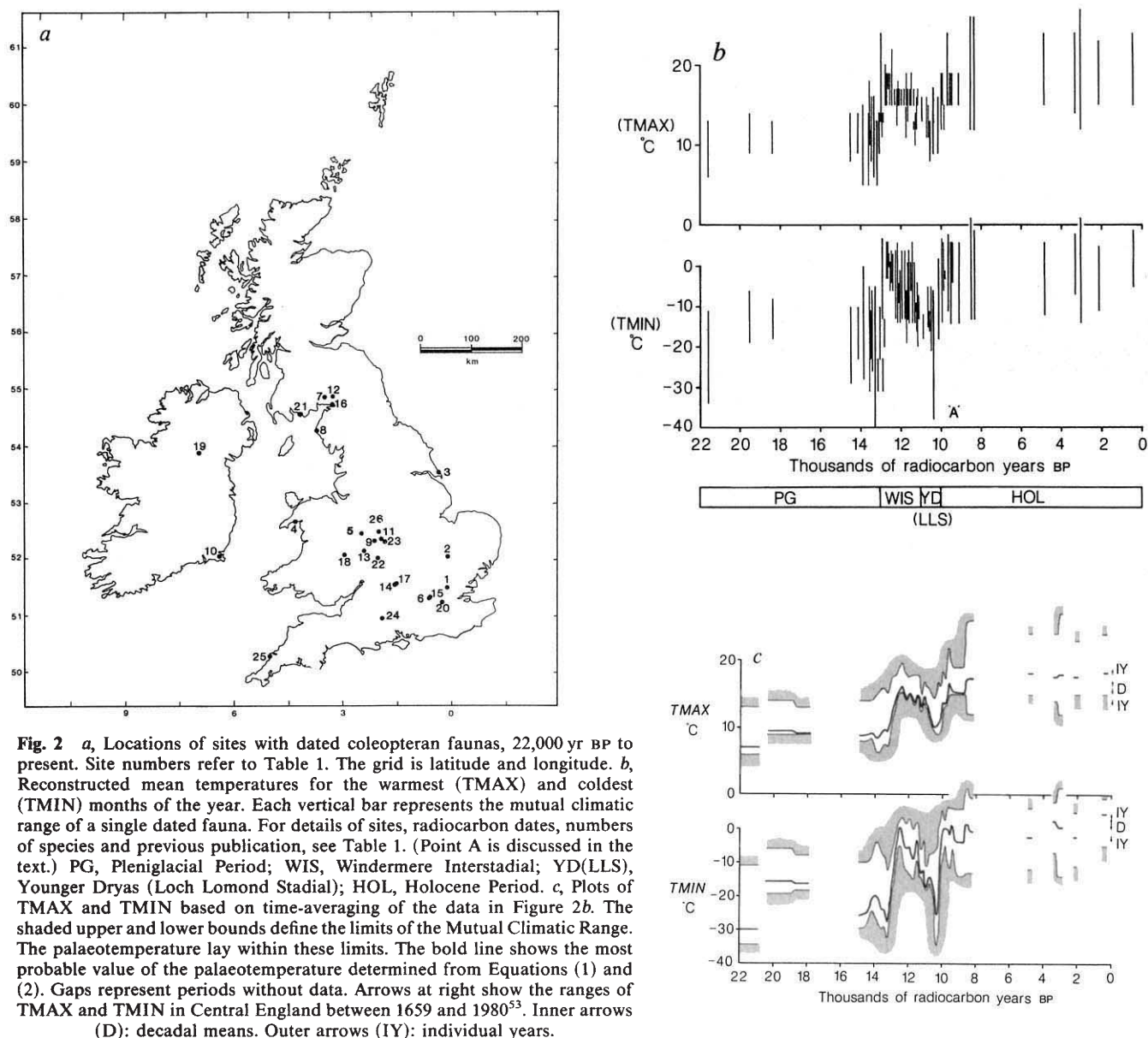


Fig. 2 *a*, Locations of sites with dated coleopteran faunas, 22,000 yr BP to present. Site numbers refer to Table 1. The grid is latitude and longitude. *b*, Reconstructed mean temperatures for the warmest (TMAX) and coldest (TMIN) months of the year. Each vertical bar represents the mutual climatic range of a single dated fauna. For details of sites, radiocarbon dates, numbers of species and previous publication, see Table 1. (Point A is discussed in the text.) PG, Pleniglacial Period; WIS, Windermere Interstadial; YD (LLS), Younger Dryas (Loch Lomond Stadial); HOL, Holocene Period. *c*, Plots of TMAX and TMIN based on time-averaging of the data in Figure 2*b*. The shaded upper and lower bounds define the limits of the Mutual Climatic Range. The palaeotemperature lay within these limits. The bold line shows the most probable value of the palaeotemperature determined from Equations (1) and (2). Gaps represent periods without data. Arrows at right show the ranges of TMAX and TMIN in Central England between 1659 and 1980⁵³. Inner arrows (D): decadal means. Outer arrows (IY): individual years.

probable value of temperature at any time is given by the bold line, and it is extremely unlikely that the actual temperature fell more than one or two °C outside the range shown by the shaded lines.

Rates and timing of climatic changes

The record of summer temperatures and climatic change shown in Fig. 2 is in substantial agreement with earlier interpretations based upon *Coleoptera*^{5,11,12}. However, the method described here has enabled the reconstruction of a detailed series of winter and mean annual temperatures. Figure 2*c* shows variations in temperature on two scales. First, and most important, are the extremely rapid major warmings at ~13,000 and ~10,000 yr BP and the prolonged cooling trend from 12,500 to 10,500 yr BP. There are also a number of minor oscillations such as the brief periods of warming around 11,300 yr BP and between 10,000 and 8,000 yr BP. Some of these may be significant but it is likely that most are the products of the arbitrary age distribution in the sites sampled.

During the Pleniglacial (22,000–18,000 yr BP, see Fig. 2*b* for definition of chronostratigraphic terms) summer temperatures were below 10 °C and the coldest winter month around –16 °C

or below. There is some suggestion on Fig. 2 that the winters were somewhat warmer around the time of maximum expansion of the British ice-sheet (20,000–18,000 yr BP) than either before or afterwards. This would be consistent with heavier snowfall.

By 14,500 yr BP the ice-sheet was in retreat or had vanished altogether in western England and Wales, and had disappeared in Scotland by ~13,000 yr BP¹³. From 14,500 until just before 13,000 yr BP the British climate was as cold as during the Pleniglacial, with the coldest winter months between –20 and –25 °C. The climate was much more continental than today, with a temperature range between warmest and coldest months of ~30–35 °C compared with 14 °C at present. Such a cold and continental thermal regime is found today in north-east European Russia and north-west Siberia. Britain today owes its mild winters to its proximity to a warm ocean. Although sea surface temperatures in the Atlantic due west of Britain are known to have been ~8 °C cooler in the summers of 14,000 years ago than today¹⁴, this cannot account for a 20 °C cooling of the British winter. The severity of the winters, and the continentality of the climate, could be explained by extensive and continuous sea ice on the Atlantic in winter. This suggestion has already been made by Ruddiman and McIntyre¹ on the

grounds of reduced ocean productivity occurring at the same time as the maximum rate of volumetric melting of glacier ice, as indicated by the isotopic composition of planktonic foraminifera. Thus, Fig. 2 gives support to Ruddiman and McIntyre's interpretation.

The period of cold climate was followed by a rapid warming of 7–8 °C in summer and ~25 °C in winter. The time-averaged data of Fig. 2c suggest that this took place over 800 years from 13,300 to 12,500 yr BP, although the raw data of Fig. 2b suggest that the warming may have commenced as late as 13,000 and been complete by 12,700 yr BP. The timing of this event agrees very well with the sudden change from a polar to a sub-polar/transitional foraminiferal assemblage which occurred at 13,300 ± 700 BP in core CH 73139C, 400 km west of Ireland¹⁴. This faunal change marks the passage of the polar front in surface waters as it migrated from its glacial position off the coast of Portugal to a position between Iceland and Greenland¹. Detailed examination of the data used in Fig. 2 shows no evidence that the warming was any later in Scotland than in England or Wales. This suggests that the polar front migrated quickly, at least through the latitudes covered by our data.

By 12,500 yr BP the climate of England and Wales was as warm as at the present day, although still slightly more continental, with the warmest summer month reaching 17 °C, and the coldest winter month reaching 0–1 °C. No sooner had this peak of warmth been reached, however, than a cooling set in from 12,500 to 12,000 yr BP. This was followed by a 500 year period in which summer temperatures remained fairly constant. In contrast, the temperature of the coldest winter month decreased from ~–5 °C at 12,000 yr BP to ~–17 °C at 10,500 yr BP. The minor oscillations shown on Fig. 2c during this cooling may not be significant. Summer temperatures also cooled from ~11,400 yr BP onwards, reaching a minimum of ~10 °C at 10,500 yr BP. Finally, a second major warming commenced after 10,500 BP. By 9,800 yr BP the climate was as warm as it had been at the peak of the Windermere Interstadial 2,500 years earlier.

This climatic history correlates closely with the movements of the polar front reconstructed by Ruddiman and McIntyre¹. In particular, the cold winters of the Younger Dryas (11,000–10,000 yr BP) suggest increased sea ice off the west coast of Britain and Ireland. Although Fig. 2c shows that the coldest winters of the Younger Dryas may have approached those of the period before 13,000 yr BP in severity, this is largely the effect of a single point ('A' on Fig. 2b) with a large MCR. Temperatures of –15 °C to –20 °C are more probable for the coldest part of the Younger Dryas. Ruddiman and McIntyre¹ consider the sea ice cover to have been less extensive in the Younger Dryas than in the earlier cold period before 13,000 yr BP and this may be reflected in the indication of a less continental Younger Dryas climate with slightly warmer winters. They also suggest that icebergs bearing volcanic ash drifted south-eastwards from west of Iceland towards the Bay of Biscay. Such a circulation in surface waters would also favour the accumulation of sea ice off the British coast.

Despite the good agreement between the British climatic record and the movements of the polar front¹, some discrepancies remain. The peak of the Windermere Interstadial was short-lived and during the cooling which followed (12,500–12,000 yr BP) the polar front lay far to the north of Britain. Thus, this cooling cannot have been a simple expression of movements of the polar front. A possible explanation lies in Jansen and Erlenkeuser's¹⁵ study of the palaeoceanography of the Norwegian sea. They find that circulation like that of the present day, with deep water being formed in the Norwegian sea and flowing out into the Atlantic at intermediate depth across the Iceland-Faroes Ridge, did not begin until the start of the Younger Dryas. Before that time the circulation appears to have been reversed with Atlantic intermediate water flowing into the Norwegian sea along the upper continental slope. This pattern was established

fairly suddenly at ~12,000 yr BP, although the time control on the cores studied by Jansen and Erlenkeuser is not good. Nevertheless, if correct, the proposed circulation between 12,000 and 11,000 yr BP would imply that cold surface waters flowed out of the Norwegian sea and could have cooled the ocean west of Britain. In this context we may also note the similarity in timing between the changes in atmospheric CO₂ recorded in the Dye 3 ice core¹⁶ and both the major warming at 13,000 yr BP and the Younger Dryas cooling in Britain. However the ice core shows approximately constant CO₂ levels in the interval 13,000–11,000 yr BP. Since atmospheric CO₂ should be closely correlated with global mean temperature through the greenhouse effect, this again suggests that the cooling in Britain after the peak of the Windermere Interstadial had a local cause in the north-east Atlantic, not a global one.

Rates of climatic change can be estimated from the "most probable" temperature curves in Fig. 2c. The major warming events involved changes of mean annual temperature (estimated as the mean of TMAX and TMIN) of 1.7 °C per century at ~10,000 yr BP and 2.6 °C per century at ~13,000 yr BP. Because of the smoothing procedure used in the construction of Fig. 2c, the true rate of change may well have been faster. By taking the individual data points on Fig. 2b (and ignoring dating uncertainties) we obtain rates of change of 2.8 and 7.2 °C per century respectively. While undoubtedly rapid, these rates of change are only between 1.5 and 6 times faster than the rate of mean northern hemispheric warming in the first half of the twentieth century¹⁷. General circulation models of climatic response to atmospheric CO₂ changes show that regional changes in high latitudes could be around four times larger than the global mean response¹⁸. Thus, the rates of the warming events in Britain at ~13,000 and ~10,000 yr BP should not be viewed with too much surprise. They are not abnormal compared with those expected from the much smaller climatic changes of the present century. However, the late glacial changes were sustained over a period 10–20 times longer than the early twentieth century warming and were of course much larger overall.

The rates of cooling between around 12,500 and 10,500 yr BP appear to be much slower than the rates of warming. The whole climatic oscillation from glacial to interstadial to glacial conditions (14,000–10,000 yr BP) shows a saw-tooth shape which seems characteristic of other records of climatic change on the 1000-year timescale. The same shape is seen in mid-Weichselian excursions of the isotopic composition of Greenland ice cores¹⁹ and in the isotopic composition of carbonates in Alpine lakes²⁰. Following Broecker's suggestion²¹ that large, rapid climatic changes might be due to the ocean-atmosphere system 'flipping' between two stable states, we note that the British palaeotemperature record is consistent with a rapid 'flip' from cold to warm conditions, but that the opposite transition must have been much more gradual. It is also noteworthy that, even ignoring the minor oscillations on Fig. 2c, the British climate was continuously changing from before 13,000 yr BP to some time after 9,800 yr BP. There is no period during this time when winter temperatures seem to have been stable for longer than a century or two. Summer temperatures may have been fairly constant for about 500 years after 12,000 yr BP but otherwise were also continuously changing.

Comparison with periglacial feature estimates

The annual march of monthly mean temperatures at the present day can be closely approximated by the sum of two sine waves, of which the more important has a 12-month period and accounts for over 95% of total variance²². In view of this, the mean annual temperature may be approximated by the mean of TMAX and TMIN. Figure 3 shows a smoothed record of mean annual temperature based on the data in Fig. 2c. These results, which are based entirely on the evidence provided by insect faunas, can be tested by comparing them with the threshold temperatures required for the formation of periglacial ice wedge casts and

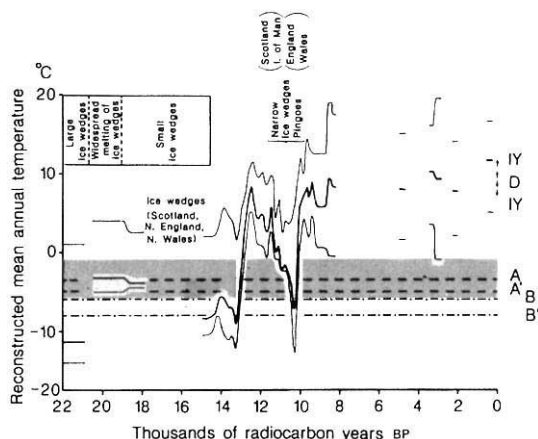


Fig. 3 Reconstructed values of mean annual temperature in England, Wales and southern Scotland, 22,000 yr BP to present, based on data in Fig. 2. The continuous fine lines show upper and lower bounds of the mutual climatic range. The bold line shows most probable value of palaeotemperature. Arrows at right show central England temperatures 1659–1980, as for Figure 2c. Shaded band shows temperature conditions within which open-system pingos can develop²⁵. Broken lines, A and A', show upper temperature limit for ice wedge development according to Washburn²⁵ and Hamilton *et al.*²⁶. Chained lines, B and B', show same limit according to Péwé²³. Box shows occurrences of ice wedges in north-west mainland Europe³⁰. Other legend shows approximate dates of periglacial features in Britain^{24,27–29,31,32}.

pingoes. Ice wedges have been variously described as forming where mean annual air temperatures are below -6 to -8 °C^{23,24} or below -5 °C²⁵, or below -3.5 °C in favourable localities such as peat soils²⁶. These limits are shown on Fig. 3 together with the approximate dating of ice wedge remains from Britain and the nearby parts of Europe. The coleopteran record indicates suitable conditions for ice wedges throughout the area of our data prior to 13,000 yr BP although the climate may have been only just cold enough around the time of maximum extent of the ice sheet (~ 20 – $18,000$ yr BP). In fact, ice wedge casts are

well known from before and after the ice sheet maximum in Britain^{24,27–29}. In Europe, Kolstrup³⁰ records a widespread melting of large ice wedges by a date no later than 19,100 yr BP after which only smaller ice wedge casts and frost cracks occur. This is in good agreement with the warming tendency in Fig. 3 between 21,500 and 19,500 yr BP and the subsequent cooling sometime between 18,300 and 14,500 BP. Narrow ice wedge casts and cracks are also known from the Younger Dryas period (11–10,000 yr BP) in Britain, but only from Scotland^{31,32} and the Isle of Man (G.R.C., unpublished data). Although Fig. 3 indicates Younger Dryas temperatures below -5 °C for 200 yr, this is largely the effect of the single value noted above ('A' on Fig. 2b) from St Bees Head on the mainland opposite the Isle of Man (site 8, Fig. 2a). Over most of the area south of Scotland, the lowest mean annual temperatures of the Younger Dryas were probably -5 °C, which is consistent with the sporadic occurrence of ice wedge casts further north.

The mean annual temperatures of -5 to -2 °C throughout most of the Younger Dryas are also consistent with the occurrence of pingo remnants in East Anglia and Wales²⁹, since active open system pingoes in Alaska range from -6 to -1 °C²⁵. Overall, there is good agreement between the evidence of periglacial features and the mean annual temperature record based on *Coleoptera*.

Conclusion

In view of its generally good agreement with the periglacial evidence and the independent verification of the MCR method, we believe the British palaeoclimate record in Fig. 2 to be the most reliable yardstick yet produced of climatic change on the oceanic margin of north-west Europe. It is better dated than either the oceanic record or ice cores. We hope that it will provide an unambiguous point of reference in the task of unravelling the precise mechanisms by which the Earth's climate changed from a glacial to an interglacial mode, mechanisms which may well have relevance to climatic change today²¹.

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- Ruddiman, W. F. & McIntyre, A. *Palaeogeogr., Palaeoclimat., Palaeoecol.* **35**, 145–214 (1981).
- Iversen, J. *Geol. Foren. Forhandl. Stock.* **66**, 463–483 (1944).
- Grichuk, V. P. in *Golotsen: K VIII kongressu-INQUA, Paris* (ed. Neishadt, M.I.) 41–57 (Izd-vo Nauka, Moscow, 1969).
- Atkinson, T. C. *et al.* in *Handbook of Holocene Palaeoecology and Palaeohydrology* (ed. Berglund, B. E.) 851–858 (Wiley-Interscience, 1986).
- Coope, G. R. *Phil. Trans. R. Soc. B* **280**, 313–348 (1977).
- Hengeveld, R. J. *Biogeogr.* **12**, 389–411 (1985).
- Hansen, V. *et al.* *Catalogus Coleopterorum Fennoscandiae et Daniae* (Entomological Society, Zoological Institute, Lund, Sweden, 1960).
- Coope, G. R. & Brophy, J. A. *Boreas* **1**, 97–142 (1972).
- Coope, G. R. & Joachim, M. J. in *Studies in the Lateglacial of Northwest Europe* (eds Lowe, J. J. *et al.*) 55–68 (Pergamon, Oxford, 1980).
- Coope, G. R. in *Ice Ages: Ancient and Modern* (eds Wright, A. E. & Moseley, F.) 153–168 (Seel House, Liverpool, 1975).
- Coope, G. R., Morgan, A. & Osborne, P. J. *Palaeogeogr., Palaeoclimat., Palaeoecol.* **10**, 87–101 (1971).
- Lowe, J. J. & Walker, M. J. C. *Reconstructing Quaternary Environments*, 332–335 (Longman, London, 1984).
- Duplessy, J. C. *et al.* *Palaeogeogr., Palaeoclimat., Palaeoecol.* **35**, 121–144 (1981).
- Jansen, E. & Erlenkeuser, H. *Palaeogeogr., Palaeoclimat., Palaeoecol.* **49**, 189–206 (1985).
- Oeschger, H. *et al.* *Am. geophys. Un. Monogr. Ser.* **29** (M. Ewing Symp. 3), 229–306 (1984).
- Jones, P. D. *et al.* *J. Clim. appl. Met.* **25**, 161–179 (1986).
- Manabe, S. & Stouffer, R. J. *J. geophys. Res.* **85**, 5529–5554 (1980).
- Dansgaard, W. *et al.* *Am. geophys. Un. Monogr. Ser.* **29** (M. Ewing Symp. 3), 288–98 (1984).
- Siegenthaler, U. *et al.* *Ann. Glaciol.* **5**, 149–152 (1984).
- Broecker, W. S., Peteet, D. M. & Rind, D. *Nature* **315**, 21–26 (1985).
- Trenberth, K. E. *Bull. Am. meteor. Soc.* **64**, 1276–1282 (1983).
- Péwé, T. L. *Biol. Periglac.* **15**, 65–73 (1966).
- Williams, R. B. G. in *Ice Ages: Ancient and Modern* (eds Wright, A. E. & Moseley, F.) 95–120 (Seel House, Liverpool, 1975).
- Washburn, A. L. *Geocryology: A Survey of Periglacial Processes and Environments*, 133–141, 283 (Arnold, London, 1979).

- Hamilton, T. D., Ager, T. A. & Robinson, S. W. *Arctic and Alpine Research* **15**, 157–168 (1983).
- Seddon, M. B. & Holyoak, D. T. *Proc. geol. Assoc.* **96**, 53–72 (1985).
- Sissons, J. B. *The Geomorphology of the British Isles: Scotland*, 110–111 (Methuen, London, 1976).
- Watson, E. *Phil. Trans. R. Soc. Lond. B* **280**, 183–198 (1977).
- Kolstrup, E. *Meded. Rijks. Geol. Dienst* **32–15**, 181–253 (1980).
- Sissons, J. B. *Scott. J. Geol.* **10**, 34–37 (1974).
- Sissons, J. B. in *Studies in the Scottish Lateglacial Environment* (eds Gray, J. M. & Lowe, J. J.) 45–59 (Pergamon, Oxford, 1977).
- Coope, G. R. & Tallon, P. W. J. *Quat. Newslett.* **40**, 7–10 (1983).
- Coope, G. R. *Geol. Magn.* **105**, 482–486 (1968).
- Penny, L. F., Coope, G. R. & Catt, J. A. *Nature* **224**, 65–67 (1969).
- Morgan, A. Univ. Birmingham, 111–126 (1970).
- Coope, G. R. *Quat. Newslett.* **38**, 1–6 (1982).
- Bishop, W. W. & Coope, R. E. in *Studies in the Scottish Lateglacial Environment* (eds Gray, J. M. & Lowe, J. J.) 61–88 (Pergamon, Oxford, 1977).
- Osborne, P. J. *Boreas* **9**, 139–147 (1980).
- Coope, G. R. in Colhoun, E. A. & Mitchell, G. F. *Proc. Roy. Irish Acad.* **71B**, 211–245 (1971).
- Osborne, P. J. *Proc. Coventry District nat. hist. Soc.* **4**, 209–13 (1973).
- Shotton, F. W. & Coope, G. R. *Proc. geol. Assoc.* **94**, 33–44 (1983).
- Coope, G. R. in *Field Guide to the Oxford Region* (ed. Roe, D.), 20–23 (Quat. Research Assoc., London, 1976).
- Shotton, F. W., Williams, R. E. G. & Johnson, A. S. *Radiocarbon* **16**, 285–303 (1974).
- Coope, G. R., Dickson, J. H., McCutcheon, J. A. & Mitchell, G. F. *Proc. R. Irish Acad.* **79B**, 63–85 (1979).
- Peake, D. S. & Osborne, P. J. *Proc. Croydon nat. hist. Sci. Soc.* **14**, 145–176 (1971).
- Osborne, P. J. *Quat. Res.* **4**, 471–486 (1974).
- Shotton, F. W., Osborne, P. J. & Greig, J. R. A. *Proc. Coventry nat. hist. Sci. Soc.* **5**, 19–32 (1977).
- Kelly, M. & Osborne, P. J. *Proc. Linn. Soc. Lond.* **176**, 37 (1964).
- Osborne, P. J. *J. animal Ecol.* **38**, 555–66 (1969).
- Clarke, B. B., Shotton, F. W., Strachan, I. & Osborne, P. J. *Trans. R. geol. Soc. Cornwall* **20**, 275–285 (1974).
- Osborne, P. J. *Brit. archaeol. Repts.* **61**, 85–109 (1979).
- Manley, G. Q. *J. R. met. Soc.* **100**, 389–405 (1974).

Gene transfer and molecular cloning of the rat nerve growth factor receptor

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The amino-acid sequence of one form of the receptor for nerve growth factor defines a new class of growth factor receptors.

ALTHOUGH nerve growth factor (NGF) was the first growth factor to be described¹ and its physiologically relevant flow from target tissue to neuronal cell body is well characterized², much less is known about the transduction of signals from the NGF receptor (NGFR) or the intracellular pathways involved in mediating the functions of NGF than is the case for many mitogenic growth factors, neurotransmitters or hormones. It is known that NGF, which is required for the development of sympathetic and some sensory neurons as well as their viability in adulthood^{2,3}, is secreted by the target tissue near the nerve terminal, binds to a receptor and is internalized by receptor-mediated endocytosis^{4,5}. The internalized NGF-containing vesicles are retrogradely transported along microtubules to the cell body⁶. Although the NGF is delivered intact and biologically active to the cell body it is quite rapidly degraded in the lysosomes^{7,8}. Somewhere in this pathway the intracellular signals that are key to functions of NGF are generated.

A better understanding of what these signals are and how they are generated is likely to follow from further knowledge of the receptor or receptors for NGF. Two apparent types of NGFR exist on the NGF-responsive neurons and in PC12 cells. The major population has a lower affinity for NGF ($K_d \sim 1$ nM) and releases NGF rapidly after binding, compared to the minor population whose affinity is two orders of magnitude higher and which release NGF relatively slowly⁹. For these reasons the NGFR are identified as the fast (low-affinity) NGFR and slow (high-affinity) NGFR. Occupied slow NGFR are trypsin resistant and insoluble in Triton X-100, whereas occupied fast NGFR are trypsin labile and soluble in Triton X-100⁹. Agents which cluster the receptor, such as wheat germ agglutinin¹⁰⁻¹² or antibodies to NGF¹², convert the fast type to a slow type of NGFR, suggesting a relationship between the types of NGFR¹³. The slow and fast NGFR when crosslinked with ¹²⁵I-NGF using *N*-hydroxysuccinimidyl-4-azido-benzoate (HSAB) form complexes of relative molecular mass 158,000 and 100,000 (M_r 158K and 100K) respectively, the latter containing a single receptor peptide chain¹⁴. Internalization of NGF in PC12 cells uses only the slow NGFR^{15,16} but both types of NGFR may be involved in this process in sympathetic neurons *in vivo*¹⁷.

To explore the relationship between the two NGFR further and with the ultimate aim of identifying receptor-mediated intracellular signals, the complementary DNA for the fast NGFR from the rat PC12 cells has been cloned. A two-step approach was used. First, using genomic-DNA-mediated gene transfer, a set of mouse cell lines that express the rat fast NGFR were produced. A cDNA clone that coded for the rat fast NGFR was then isolated based on the difference in gene expression in the newly created NGFR⁺ cell lines and the original mouse cell line. The 426 amino-acid sequence deduced from the cDNA sequence shows that the fast NGFR is unrelated to any other growth factor receptor. Indirect evidence suggests that the slow NGFR comprises the fast NGFR moiety together with a second subunit.

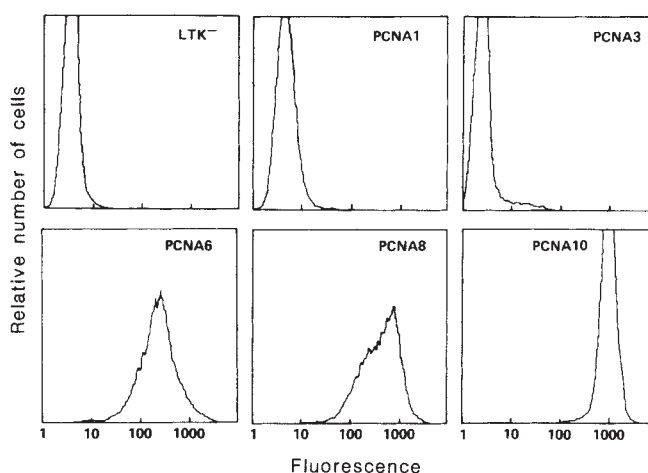


Fig. 1 Amplification of the expression of NGFR in the PCNA cell line. The first panel shows a control staining of LTK⁻ cells. The remaining panels show the amplification of NGFR expression in the PCNA cells after repeated rounds of sorting. The number attached to the cell line name indicates the number of times the cells had been sorted.

Methods. LTK⁻ cells (10^6) were transfected with 20 μ g of PC12 genomic DNA and 1 μ g of pCHTK using a modified CaPO₄ procedure of Wigler *et al.*^{18,19}. Stable transfectants were selected by growth in hypoxanthine, aminopterin and thymidine (HAT) medium¹⁹. After 2–3 weeks of HAT selection the transfectants were harvested in phosphate-buffered saline (PBS) with 1 mM EDTA. Cells (10^6) were then collected by centrifugation and resuspended in 50 μ l of staining medium¹⁹ containing 1 μ g of the anti-NGFR monoclonal antibody, MC192 (ref. 20). After 30 min of incubation at 4°C the cells were washed once with 200 μ l of staining medium and resuspended in 50 μ l of 4°C staining medium plus 1 μ g of fluorescein-conjugated goat anti-mouse IgG and IgM (Tago, Inc.). After 20 min an equal volume of staining medium (2 ng propidium iodide ml⁻¹) was added to detect dead cells. Ten minutes later the cells were washed twice with 200 μ l of staining medium. The stained cells were then resuspended in 200 μ l of staining medium, passed through a 50 μ m screen and sorted on a FACS. The most intensely staining cells (~1%) were collected and grown for 2–3 weeks in HAT medium at which time there were enough cells to stain and sort again.

Transfection and amplification

Mouse LTK⁻ cells were cotransfected with high-molecular-mass rat PC12 pheochromocytoma genomic DNA (100–200 kilobases (kb) in length) and plasmid (pCHTK) containing the chicken thymidine kinase gene^{18,19}, and LTK⁺ cells selected by growth in HAT medium¹⁸. To detect transfectants that expressed the rat fast NGFR the LTK⁺ cells were stained with MC192, a mouse monoclonal antibody against the fast NGFR²⁰, and fluorescein-conjugated goat anti-mouse immunoglobulin G

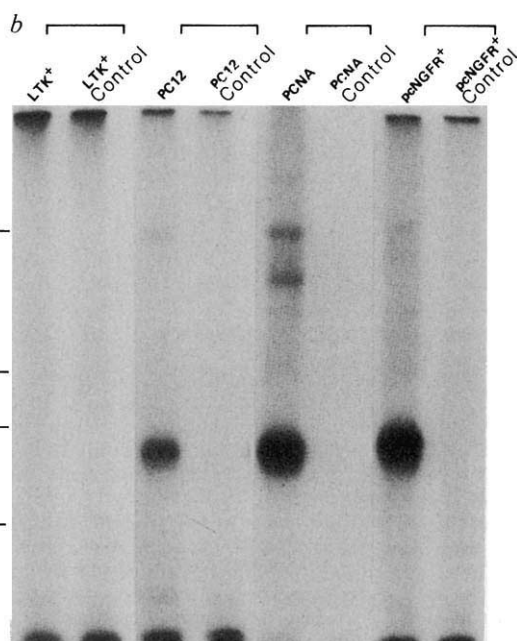
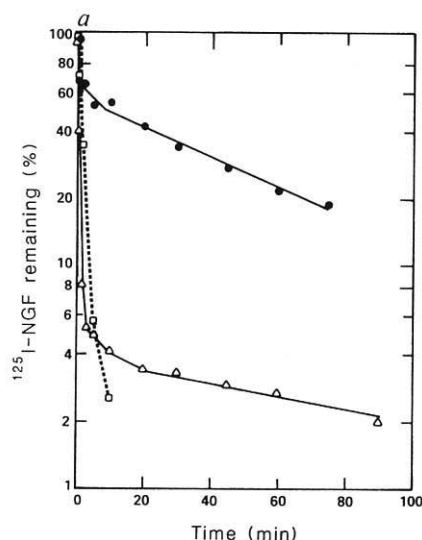


Fig. 2 Characterization of the NGFR in PC12 and transfected cell lines. *a*, Dissociation kinetics of PC12 (●), PCNA (△) and pcNGFR⁺ (□) cells. Results are expressed as the percentage of the initial specific binding before dissociation. PCNA and pcNGFR⁺ cells were washed once, harvested in Ca²⁺ Mg²⁺-free PBS, 1 mM EDTA, pH 7.4, and washed in 10 mM HEPES/Krebs-Ringer saline, pH 7.4 (Krebs-Ringer), containing 1 mg ml⁻¹ each of glucose and bovine serum albumin (binding buffer). PC12 cells were harvested by trituration and washed four times in binding buffer. PC12 cells (2×10^6 ml⁻¹), PCNA cells (0.5×10^6 ml⁻¹), and pcNGFR⁺ cells (2×10^6 ml⁻¹) were incubated with 100 pM [¹²⁵I]-NGF in 1.5 ml of binding buffer for 60 min at 22 °C, dissociation initiated by addition of 500–1000-fold unlabelled NGF at 37 °C, and residual binding assayed⁹. *b*, Determination of the size (*M_r*) of the NGFR. Lanes 1, 3, 5 and 7 are analyses of immunoprecipitates obtained with the MC192 antibody from LTK⁺, PC12, PCNA, and pcNGFR⁺ cell surfaces respectively. Control lanes are of immunoprecipitates with anti-agrin. The cells were harvested as described above, except that glucose and BSA were omitted. LTK⁺ cells (12×10^6), PC12 cells (14×10^6), PCNA cells (13×10^6) and pcNGFR⁺ cells (11×10^6) were surface radioiodinated⁴² and solubilized in 1 ml of 0.5% Nonidet P40 Krebs-Ringer containing a mixture of 10 protease inhibitors. After one hour on ice, nuclei were removed by centrifugation at 4 °C. To one half of each supernatant 5–6 µg of anti-receptor MC192 antibody was added and the suspension was incubated at 4 °C with mixing for 12 h. The remainder (controls) received 10 µg of antibody raised against agrin (gift of Dr U. J. McMahan). After centrifugation at 10,000g for 10 min, 50 µl of a suspension of goat anti-mouse antibodies coupled to agarose were added to each supernatant and incubated at 4 °C with mixing for an additional 12 h. Immunoprecipitates were collected and washed four times with 0.5% NP40. After removal of excess NP40, immunoprecipitates were stored at –20 °C until analysed by SDS-polyacrylamide gel electrophoresis⁴³.

(IgG). Positive cells were then isolated using a fluorescence-activated cell sorter (FACS).

In eight independent transfections, six positive cell lines were obtained. The fluorescence intensity of five of them was similar to that of PC12 cells. The sixth cell line had a heterogeneous, unstable phenotype. With repeated growth in HAT medium and sorting on the FACS the average intensity continued to increase. After 10 rounds of sorting and regrowth, a stable cell line, PCNA, was obtained (Fig. 1).

Analysis of the transfected L cell lines

The transfectants were analysed by several methods to ensure that they expressed PC12 NGFR and to determine the form of receptor. The rate of dissociation of [¹²⁵I]-NGF from PCNA cells showed that it expressed only fast NGFR (Fig. 2*a*). Similar results were obtained with all the other transfectants. Scatchard analysis of the steady-state binding of PCNA cells revealed a single class of NGFR with a *K_d* (2.3 nM) identical to that of PC12 fast NGFR, and a number of NGFR (2.3×10^6 per cell) more than 10-fold greater than on PC12 cells. Analysis of the immunoprecipitates of [¹²⁵I]-labelled PC12 and PCNA cell-surface proteins with the MC192 antibody showed the same

major protein of *M_r* 83K (Fig. 2*b*), the expected size of the fast NGFR^{14,21}. Although not apparent in this figure, the immunoprecipitate actually comprises two species with *M_r* s of 82K and 85K. The minor 200K species observed in these analyses is probably a disulphide-linked oligomer of the 83K species^{22,23}. Finally, only the 100 K crosslinked, fast NGFR-NGF complex was observed in PCNA cells after crosslinking with HSAB. From these data we conclude that transfer of the rat fast NGFR into mouse L cells was achieved.

Cloning of the rat fast NGFR cDNA

The rat fast NGFR gene was rescued by relying on the fact that the NGFR⁺ L-cell transfectants express fast NGFR mRNA whereas the L cells do not. In brief, a fast-NGFR-enriched probe was made by subtracting ³²P-labelled PCNA cDNA (made, in turn, from PCNA poly(A)⁺ RNA) with a 20-fold excess of LTK⁺ poly(A)⁺ RNA²⁴ to remove constitutive LTK⁺ cDNA. The remaining ³²P-labelled cDNA (10% of total cDNA) was used to screen 60,000 colonies of a PCNA cDNA library in pUC9^{25–27}. The expression of the fast NGFR is very high in PCNA cells, so it seemed likely that the cDNA for this receptor would be one of the more abundant species detected with the subtracted

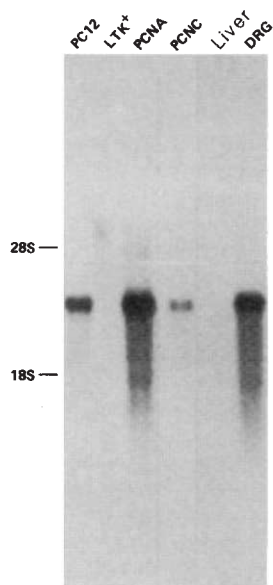


Fig. 3 Northern analysis of the NGFR cDNA clone. Total RNA⁴⁴ (10 µg) from PC12 cells, LTK⁺ cells, PCNA cells, PCNC cells, rat liver and newborn rat dorsal root ganglia (DRG) were each separated on a 0.8% formaldehyde agarose gel⁴⁵ and blotted to a nylon membrane according to the manufacturer's instructions (Hybond, Amersham Corp.) A ³²P-labelled cDNA probe (~30 × 10⁶ c.p.m.) was made from the putative NGFR cDNA clone insert (pNGFR.1), using random primers⁴⁶ and hybridized with the blot in 10 ml of 50% formamide, 5× SSPE, 5× Denhardt's, 1 mM ATP and 0.1% SDS for 3 days at 42°C. The blot was then washed 4 times for 30 min in 0.2×SSC and 0.1% SDS at 65°C and subjected to autoradiography²⁵.

probe. Based on this assumption 30 of the most intensely hybridizing colonies were picked. Rescreening reduced this number to 19. To simplify the identification of fast NGFR clones the 19 clones were separated into groups based on cross-hybridization of their inserts. From one family of clones that reacted most strongly with the subtracted probe, a clone with an insert of 3.4 kb was selected. This insert detected a common 3.7-kb message in PC12, PCNA and PCNC (another transfectant) cells and sensory (dorsal root ganglion) neurons which express fast NGFR, but not in LTK⁺ or rat liver cells which do not (Fig. 3), suggesting that it contained a fast NGFR cDNA.

The 3.4-kb clone was inserted into the simian virus 40 (SV40)-based vector, pcDL1, in sense and antisense directions and the two subclones (pcNGFR⁺ and pcNGFR⁻) independently transfected into LTK⁻ cells. The FACS analysis of both LTK⁺ cells and the transfectant with pcNGFR⁻ after HAT selection exhibited background levels of staining, whereas the transfectants with pcNGFR⁺ stained positively. The brightest 10% of these cells were collected, grown in HAT medium and analysed for fast NGFR in the same way as were the genomic DNA transfectants. Scatchard analysis showed a single class of receptors with a K_d of 3.5 nM, similar to that of fast NGFR on PC12 and PCNA cells, and ~17,000 receptors per cell. The rate of ¹²⁵I-NGF dissociation was also characteristic of the PC12 fast NGFR (Fig. 2a) and immunoprecipitation of ¹²⁵I-labelled surface proteins showed the 83K species, confirming that the fast NGFR in these L cells transfected with pcNGFR⁺ is of the correct size (Fig. 2b). We conclude that the 3.4-kb clone, now identified as pNGFR.1, contains the rat fast NGFR cDNA.

Nucleotide sequence of fast NGFR cDNA

The nucleotide sequence of the fast NGFR cDNA, with its predicted amino-acid sequence, is shown in Fig. 5a. A model of the fast NGFR protein and a restriction map of the pNGFR.1

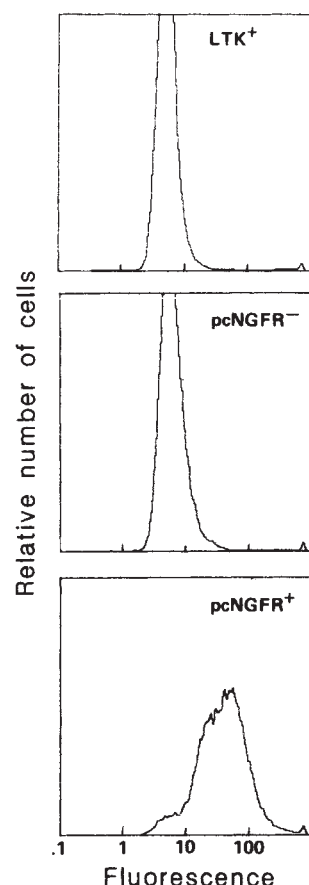


Fig. 4 Transfection of the full length NGFR cDNA in an expression vector into mouse L cells. The complete insert from the putative NGFR cDNA clone, pNGFR.1, was excised with *Eco*RI and *Hind*III and blunt-ended with the large fragment of *Escherichia coli* DNA Pol I²⁵. The insert was then cloned in either orientation in front of the SV40 early gene promoter of pcDL1 at the *Eco*RI site which was also blunt-ended. Plasmid pcDL1, which was made from the Okayama and Berg cDNA cloning vectors pcDV1 and pcL1, was a gift of Dr Frank Lee (DNAX). An *Eco*RI site was inserted into the normal cDNA cloning site. The pcDL1-NGFR constructs (1 µg), pcNGFR⁺ and pcNGFR⁻, were independently transfected with 1 µg of pCHTK and 20 µg of carrier DNA (isolated from A875 cells) into LTK⁻ cells^{18,19}. LTK⁻ cells transfected with A875 genomic DNA and pCHTK served as a control. After HAT selection the stable transfectants were stained with MC192 and FITC conjugated goat anti-mouse IgG and IgM¹⁹. The stained cells were then analysed using the FACS.

insert are given in Fig. 6. The first start codon is 114 bases from the 5' end. The open reading frame continues for 1,275 bases which results in a precursor peptide with M_r 45,432. Preliminary amino-acid sequencing of the fast NGFR, purified from the PCNA cells, indicates that 29 N-terminal amino-acid residues are removed, leaving a lysine at the amino terminus of the mature receptor protein (T.P.M., M.J.R. & E.M.S., manuscript in preparation). The NGFR expressed by the human melanoma cell line, A875 has a nearly identical N-terminal sequence²⁸. Cleavage of this signal peptide results in a mature peptide containing 396 amino-acid residues with M_r 42,478.

Analysis of the hydropathy of the predicted fast NGFR peptide²⁹ suggests only one plasma membrane spanning domain (residues 225–249). The amino-terminal, probably extracellular, domain of the protein, comprising 222 amino-acid residues, is very rich in cysteine residues, has 2 putative sites for N-linked glycosylation³⁰ and is very acidic. It contains four repeating elements in which the positions of the cysteine residues are highly conserved (Fig. 5b). The intracellular domain of 151

a



b



Fig. 5 a, Nucleic acid sequence of pNGFR.1 cDNA and the predicted amino-acid sequence of the rat fast NGFR. The nucleotides are numbered on the left and the amino-acid residues are numbered above the sequence. Arrows, two possible sites of asparagine-linked glycosylation; round-ended box, putative membrane-spanning domain. The cysteine residues are in rectangles and the four repeating elements are underlined. The sequence was obtained on both strands primarily using a shotgun approach⁴⁷ and dideoxy sequencing in M13mp19^{48,49}. Areas of ambiguity were determined by sequencing convenient restriction fragments by the Maxam and Gilbert method⁵⁰. The sequence of the remaining 5' non-coding region and confirmation of the fast NGFA sequence awaits the cloning and sequencing of the genomic gene. b, Comparison of the sequences of the four cysteine-rich repeating elements. The cysteines are boxed in and amino-acid residues which are conserved are in a bold type. Dashes, gaps inserted to maximize similarity.

amino acids, on the other hand, has only 3 cysteines and is essentially neutral in charge. It also lacks the consensus sequence of an ATP-binding site, a characteristic of both the tyrosine and serine or threonine kinases³¹. From its nucleotide and amino-acid sequence the fast NGFR appears to be a unique protein. Comparison with the NBR protein data bank as well as the EMBL and Genbank DNA libraries revealed no significant homologies with any other known protein in either nucleotide or amino-acid sequence. This lack of homology, especially with other growth factor receptors or oncogenes, is perhaps not surprising as NGF is a survival and differentiation factor and not a mitogen.

The difference in size between the core protein of the fast NGFR (42,478) and the mature receptor (~83 K) presumably resides in the carbohydrate moieties on one or both of the asparagine residues available for N-linked glycosylation and on one or more of the available O-linked glycosylation sites.

Comparison with other receptors

Although the fast NGFR is a unique protein it has some features in common with other receptors. Repeating elements in the cysteine-rich region in the extracellular domain are also found in other peptide-binding receptors such as the epidermal growth

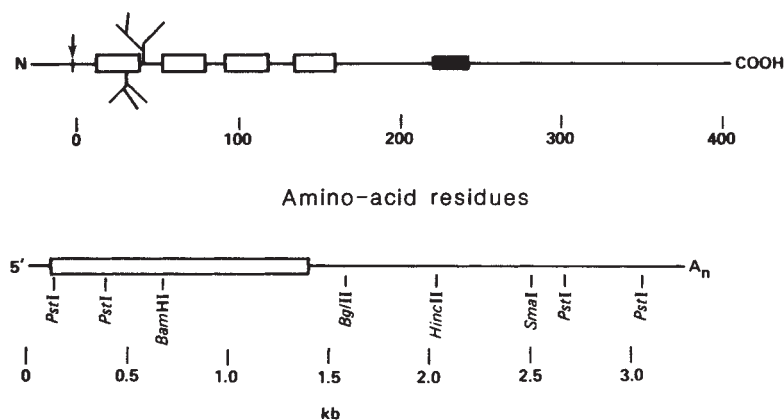


Fig. 6 Restriction map and model of the fast NGFR protein. Bottom, a representation of the transcript and its restriction map. Box, translated portion of the fast NGFR transcript; line, the untranslated portion of the cDNA. Above, a model of the fast NGFR protein. Arrow, site at which the signal peptide is cleaved; black boxes, putative membrane-spanning domain; white box, cysteine repeating elements. The two possible sites for *N*-linked glycosylation are marked by arbitrarily branched side chains.

factor (EGF)³², insulin^{33,34} and low density lipoprotein (LDL)³⁵ receptors. In addition, the EGF, PDGF³⁶ and LDL receptors, and one half of the insulin receptors, like the fast NGFR, have single membrane-spanning domains. The cytoplasmic domain is much shorter than the cytoplasmic domains of the receptors (EGF, PDGF, insulin and *c-fms*³⁷ receptors) with endogenous tyrosine kinase activity. It is more comparable in size to the cytoplasmic domains of the β -adrenergic (C-terminal segment)³⁸ and LDL receptors which also lack this activity. As in the β -adrenergic receptor the cytoplasmic domain of the fast NGFR is rich in serine and threonine residues. Some of these residues in β -adrenergic receptor are phosphorylated by a kinase only when an agonist is bound to the receptor³⁹. Phosphorylation of the human fast NGFR receptor on serine and threonine residues has been reported; however the phosphorylation appears to be insensitive to NGF²¹. The fast NGFR is one of the smaller receptors to be characterized. Thus far only the interleukin-2 (IL-2) receptor, containing 251 amino-acid residues, is smaller^{40,41}. This receptor is similar to the fast NGFR in that approximately half its apparent mass is carbohydrate and it displays two affinities of binding for its ligand IL-2.

The most distinctive feature of the fast NGFR compared to other growth factor receptors is the lack of an ATP binding site in the cytoplasmic domain, suggesting that the binding of NGF to the fast NGFR does not activate an endogenous kinase in

the receptor. The key to the signal transduction mechanism of NGF therefore lies in the difference between the structures of the fast and the slow NGFR. Several models can be proposed to account for this difference: (1) they result from differential splicing of a common precursor mRNA; (2) they arise from separate but homologous genes; (3) they share a common NGF-binding subunit (namely the fast NGFR) or (4) they have no relation other than their ability to bind NGF. The finding that the fast NGFR cDNA hybridizes to a single mRNA species in cells (PC12 and sensory neurons) that express both types of receptor argues against the first two possibilities. It seems likely, therefore that the two receptors share the fast NGFR as a common binding subunit and that the slow NGFR contains a second subunit of $M_r \sim 60K$. The involvement of a *ras*-like gene product in the pathway of action of NGF raises the possibility that the second subunit is one of the family of G proteins.

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Note added in proof: Since submission of this article a very similar sequence has been reported for the human low-affinity NGFR (D. Johnson *et al. Cell* 47, 545-554; 1986).

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- Levi-Montalcini, R. & Hamburger, V. *J. exp. Zool.* **116**, 321-363 (1951).
- Thoenen, H. & Barde, Y.-A. *Physiol. Rev.* **60**, 1284-1334 (1980).
- Levi-Montalcini, R. & Angeletti, P. U. *Physiol. Rev.* **48**, 534-569 (1968).
- Schwab, M. E., Heumann, R. & Thoenen, H. *Cold Spring Harb. Symp. quant. Biol.* **46**, 125-134 (1982).
- Lay, P. G. & Shooter, E. M. *J. biol. Chem.* **258**, 3012-3018 (1983).
- Schnapf, B. J., Vale, R. D., Sheetz, M. P. & Reese, T. S. *Cell* **40**, 455-462 (1985).
- Korsching, S. & Thoenen, H. *Neurosci. Lett.* **39**, 1-4 (1983).
- Korsching, S. & Thoenen, H. *J. Neurosci.* **5**, 1058-1061 (1985).
- Vale, R. D. & Shooter, E. M. *Meth. Enzym.* **109**, 21-39 (1985).
- Vale, R. D. & Shooter, E. M. *J. Cell Biol.* **94**, 710-717 (1982).
- Grob, P. M. & Bothwell, M. A. *J. biol. Chem.* **258**, 14136-14143 (1983).
- Vale, R. D. & Shooter, E. M. *Biochemistry* **22**, 5022-5028 (1983).
- Vale, R. D. & Shooter, E. M. in *Handbook of Physiology* (ed. Cowan, W. M.), (Waverly Press, Baltimore, in the press).
- Hosang, M. & Shooter, E. M. *J. biol. Chem.* **260**, 655-662 (1985).
- Shooter, E. M., Yankner, B. A., Landreth, G. E. & Sutter, A. *Recent Prog. Horm. Res.* **37**, 417-446 (1981).
- Bernd, P. & Greene, L. A. *J. biol. Chem.* **259**, 15509-15516 (1984).
- Dumas, M., Schwab, M. E. & Thoenen, H. *J. Neurobiol.* **10**, 179-197 (1979).
- Wigler, M. *et al. Cell* **11**, 223-232 (1979).
- Kavathas, P. & Herzberg, L. A. *Proc. natn. Acad. Sci. U.S.A.* **80**, 524-528 (1983).
- Chandler, C. E., Parsons, L. M., Hosang, M. & Shooter, E. M. *J. biol. Chem.* **259**, 6882-6889 (1984).
- Grob, P. M., Ross, A. H., Koprowski, H. & Bothwell, M. A. *J. biol. Chem.* **260**, 8044-8049 (1985).
- Tranquilli, M., Schweitzer, J. B. & Johnson, E. M. Jr *Proc. natn. Acad. Sci. U.S.A.* **83**, 1950-1954 (1986).
- Buxser, S., Puma, P. & Johnson, G. L. *J. biol. Chem.* **260**, 1917-1926 (1985).

- Hedrick, S. M., Cohen, D. I., Nielsen, E. A. & Davis, M. M. *Nature* **308**, 149-153 (1984).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
- Huynh, T. V., Young, R. A. & Davis, R. W. in *DNA Cloning: A Practical Approach* (ed. Grover, D. M.) 49-78 (ITL, Washington D.C., 1985).
- Hanahan, D. *J. molec. Biol.* **166**, 557-580 (1983).
- Marano, N. *et al. J. Neurochem.* (in the press).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105-132 (1982).
- Hubbard, S. C. & Ivatt, R. J. A. *Rev. Biochem.* **50**, 555-583 (1981).
- Kamps, M. P., Taylor, S. S. & Sefton, B. M. *Nature* **310**, 589-591 (1985).
- Ullrich, A. *et al. Nature* **309**, 418-425 (1984).
- Ebina, Y. *et al. Cell* **40**, 747-758 (1985).
- Ullrich, A. *et al. Nature* **313**, 756-761 (1985).
- Yamamoto, T. *et al. Cell* **39**, 27-38 (1984).
- Yarden, Y. *et al. Nature* **323**, 226-232 (1986).
- Coussens, L. *et al. Nature* **320**, 277-280 (1986).
- Dixon, R. A. F. *et al. Nature* **321**, 75-79 (1986).
- Benovic, J. L., Strasser, R. H., Caron, M. G. & Lefkowitz, R. J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2797-2801 (1986).
- Leonard, W. J. *et al. Nature* **311**, 626-631 (1984).
- Nikaido, T. *et al. Nature* **311**, 631-635 (1984).
- Ledbetter, J. A., Seaman, W. E., Tsu, T. T. & Herezenberg, L. A. *J. exp. Med.* **153**, 1503-1516 (1981).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294-5299 (1979).
- Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, H. *Biochemistry* **16**, 4743-4751 (1977).
- Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6-13 (1983).
- Messing, J. *Meth. Enzym.* **101**, 20-78 (1983).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5465-5467 (1977).
- Yanisch-Perron, C., Vierira, J. & Messing, J. *Gene* **33**, 103-119 (1985).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499-560 (1980).

Can explosions generate large-scale cosmological structure?

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The explosion model is successful in explaining certain features of galaxy formation but it is not clear *a priori* whether it can account for the large-scale distribution of galaxies. In particular, recent claims to have found 'bubbly' structure raise the question of whether explosions could have generated bubbles as large as observed. We here address this question semi-quantitatively, using analytic estimates and the results of first *N*-body simulations. We conclude that, in a flat Universe, the explosion model can produce a galaxy distribution on scales of a few to a few tens of megaparsecs which is not in apparent conflict with observations provided the galaxies formed at a redshift $z \approx 5$ –10. If the Universe is 90% dominated by non-gaseous dark matter, the model provides a natural biased galaxy formation mechanism, as seems to be required to reconcile a flat cosmological model with observation.

The large-scale structure of the Universe is traditionally assumed to derive from primordial density fluctuations via the influence of gravity alone. However, it is also possible that the structure derives in part from non-gravitational processes, with a much weaker dependence on the initial conditions of the Universe. This is suggested, for example, by indications that galaxies did not form in the same way everywhere¹. Such 'biasing' seems to be required if the Universe has the critical density, as suggested by the inflationary model, and it implies that some form of astrophysical feedback must have played a role in determining the large-scale structure.

A particularly interesting type of astrophysical feedback would arise if the first objects could produce explosive energy. The idea is that the shocks generated by such explosive 'seeds' (stars or clusters or stars) could sweep up background gas into dense expanding shells, which in certain circumstances would fragment into new objects. Providing radiative processes are the dominant cooling mechanism (which applies for redshifts $z < 10$), one would expect the fragments to be of galactic scale.

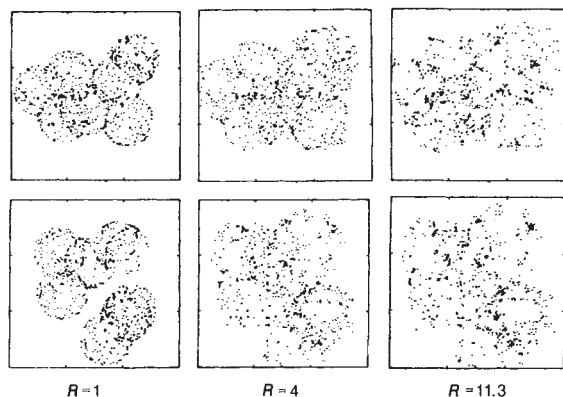


Fig. 1 Co-moving evolution (left to right) of the projected distribution of galaxies as viewed from two orthogonal directions (top and bottom) in a simulation with $\Omega_g = 0.1$.

Since the shells could have much more mass than the original seeds, this is a natural way of amplifying the scale of structure in the Universe²⁻⁹. If the shells eventually overlap, either before or after they have fragmented, it would also be a natural way of generating 'filaments' and 'voids'. In this paper we study this idea in some detail. First we discuss constraints on the maximum size of the individual shells and then use *N*-body simulations to study the effects of shell-shell interactions.

The first constraint on the shell size derives from the requirement that the amplification factor η (the ratio of the final shell mass M_{shell} to the seed mass M_{seed}) be large enough. As η decreases with increasing seed mass ($\eta \propto M_{\text{seed}}^{-0.4}$), whereas M_{shell} increases ($M_{\text{shell}} \propto M_{\text{seed}}^{0.6} \propto \eta^{-1.5}$), this places an upper limit on M_{shell} . In terms of co-moving radius normalized to the present epoch, one can show that a given value of η corresponds to a size¹⁰

$$R_{\text{shell}} = 1.7(1+z_f)^{-0.5} \left(\frac{\epsilon}{10^{-4}} \right)^{0.5} \left(\frac{\eta}{100} \right)^{-0.5} \left(\frac{\Omega_g}{0.1} \right)^{0.5} \Omega^{-1} h^{-1} \text{ Mpc} \quad (1)$$

where h is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, z_f is the redshift at which the shell fragments, ϵ is the efficiency with which the seed mass is converted into explosive energy (normalized to the value associated with supernova explosions), Ω_g is the gas density in units of the critical density (normalized to the value indicated by primordial nucleosynthesis), Ω is the total density parameter (here taken to be unity), and we assume that the explosion occurs when radiative cooling dominates. If the explosive seeds are stars which also produce metals (as is most likely), η may need to exceed 100 in order to avoid generating too much background metallicity⁴. In this case, R_{shell} can be at most a few Mpc. Although one can weaken this constraint in various ways, even the condition $\eta > 1$ imposes a limit of a few tens of Mpc.

Another constraint derives from the requirement that the shells must be able to fragment (that is they must be able to cool). If radiative cooling dominates, one can show that this gives an upper limit on the co-moving shell radius¹⁰ of

$$R_{\text{shell}} = 3.4 (1+z_f)^{-0.2} \left(\frac{\Delta}{4} \right)^{0.2} \left(\frac{\Omega_g}{0.1} \right)^{0.2} \Omega^{-0.6} h^{-0.8} \text{ Mpc} \quad (2)$$

where the factor Δ indicates how overdense the shell is relative to the background density. For an isolated shell, $\Delta \approx 4$ but it could be larger if shells fragment only when they collide (since this increases the shell density). Again one has a limit of about 10 Mpc.

The amplification and fragmentation constraints could be weakened if the shell fragments themselves explode. In this case, the fragments could produce a second shock which amplifies the scale of structure still further. Indeed, providing the shells do not overlap too soon, one could initiate a bootstrap process⁴ (or a detonation wave⁸) in which the shell size at the present epoch could be as large as

$$R_{\text{shell}} \approx 30 \left(\frac{\epsilon}{10^{-4}} \right)^{0.5} h^{-1} \text{ Mpc} \quad (3)$$

This is possible providing the cooling time is much shorter than the cosmological time. This condition may only be satisfied in the era when the Compton cooling of the microwave background dominates radiative cooling ($z > 10$) but, in any case, (3) gives a useful upper limit to the maximum shell size attainable in principle.

The crucial question is whether the explosion model can produce a galaxy distribution which resembles the observed one on large scales. The limited information so far available from redshift surveys indicates the presence of 'filamentary' or 'cell' structure, with thin, elongated 'superclusters' surrounding low density 'voids'¹¹. The apparent scale of this structure is of the

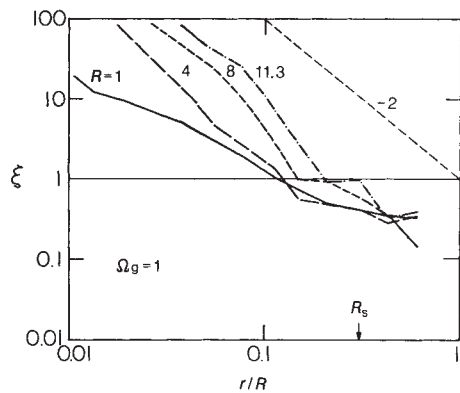


Fig. 2 Co-moving evolution of the averaged galaxy-galaxy correlation function in the scenario with $\Omega_g = 1$.

order of a few tens of megaparsecs. For example, the Local Supercluster¹² is a 'pancake' with a diameter of at least $\sim 20 h^{-1}$ Mpc and the Perseus-Pisces Supercluster¹³ is a filament with length up to $\sim 100 h^{-1}$ Mpc. Of current interest is the distribution of galaxies in a $6^\circ \times 120^\circ \times 15 h^{-1}$ Mpc slice of the Universe near Coma¹⁴; the observers claim to see 'bubbles' with a typical radius of $12.5 h^{-1}$ Mpc and one especially round bubble surrounded by part of an apparently thin smooth shell of radius $25 h^{-1}$ Mpc. A study of similar slices in the Perseus-Pisces region¹⁵, as well as deeper surveys¹⁶⁻¹⁹, also indicate the presence of filamentary structure (though not necessarily spherical 'bubbles') with a few very large voids—regions of galaxy density less than 20% of the mean—of diameter $\sim 25 h^{-1}$ Mpc. The largest known void (in Bootes¹⁹) has a radius of $\sim 30 h^{-1}$ Mpc.

If one naively interprets these voids as the interiors of the isolated shells produced in the explosion model, and the superclusters as portions of the shells themselves, then our estimates predict that the average 'void' radius would be at most $10 h^{-1}$ Mpc and the supercluster length at most $20 h^{-1}$ Mpc. These values are somewhat small in comparison with the observations. However, the size of the individual shells is not the whole story. Present day galaxies are certainly not distributed on the surfaces of isolated shells and this indicates that the shells, if they existed at all, must have overlapped and interacted with each other. In this case, the inter-shell interaction must have had an important effect on the characteristic scale of the final distribution. If galaxies form by spontaneous fragmentation before the shells significantly overlap, this interaction is mainly gravitational: the observed superclusters will form along the regions of overlap which may extend through several shells (see Fig. 1) and the resulting voids may be larger than one would naively expect (especially where large shells interact with smaller ones). If the shells overlap significantly while they are still gaseous, with galaxies forming only in the overlapping regions, the size of the resulting voids and superclusters is expected to be even larger. In this paper we consider only the first situation since it is easier to simulate numerically.

The simulations described below are intended to provide a preliminary study of some of the gravitational effects of the shell interactions. We focus on scales $1\text{--}20 h^{-1}$ Mpc. At the lower end of this range, the galaxy distribution could be strongly affected by the detailed fragmentation process within the shells; we here assume a uniform random distribution along the shell surfaces. At the high end and beyond, the structure will be partially determined by the original seed distribution; this may reflect correlations due to any primordial fluctuations (for example, cold dark matter fluctuations, metric fluctuations, grain fluctuations, or fluctuations induced by cosmic strings)²¹. Here, we examined whether one can do without such primordial fluctuations, assuming that the seeds merely have a Poisson distribution initially.

Our simulations study the evolution of a gravitating system which consists of three point-like particle components, representing galaxies (gal), gas clouds (g) and dark matter clouds (DM). This is done by means of a direct N -body code which integrates the newtonian equations of motion of N particles subject to a softened gravitational potential²². The DM particles, which contribute Ω_{DM} to the total mass density, are distributed uniformly inside a spherical volume of radius R (set to unity at the beginning of the simulation). Prior to the explosions, the gas is also assumed to be uniformly distributed, with a density parameter $\Omega_g = 1 - \Omega_{DM}$ (the total density is taken to be critical). N_s seeds are then chosen at random positions inside the volume and a sphere of radius R_s is drawn around each of them. Each sphere is assumed to be evacuated of gas, all of this gas having fragmented at redshift z_f into galaxies which are randomly distributed on its surface. The galaxies are given an initial radial expansion speed which is 20% larger than the Hubble speed at the shell radius; this approximates the property of the cosmological similarity solution which describes the evolution of the precursor gas shells⁵. (Strictly speaking, this is an overestimate, since the similarity solution is only approached asymptotically, but the error involved is not very large.) Where the shells already happen to overlap at z_f the galaxies are placed on the plane of symmetry of each overlapping region; this simulates the result of the inelastic encounter of the two gaseous shells before z_f . In this case, we suppress the velocity component normal to the plane of intersection but retain the transverse component. The gas and the DM particles start with an unperturbed Hubble flow.

In the illustrative cases presented here, we started with 8 shells of radius $R_s = 0.3 R$, each containing 100 galaxies. The volume covered by the spheres is roughly 20% of the total volume; the precise percentage is affected by the fact that we require each sphere to be entirely contained within the radius R . The masses and initial softening radii adopted for the particles are $m = 1, 5, 5$ and $\epsilon = 0.007 R, 0.035 R, 0.035 R$ for the galaxies, gas and DM, respectively. The softening parameter represents the finite size of the galaxies and clouds, and is therefore fixed in the non-co-moving coordinates where R increases with time.

The first model is a purely baryonic Universe ($\Omega_g = 1$) with no DM (except perhaps for that associated with galactic haloes). The second model assumes $\Omega_g = 0.1$ and so incorporates a dominant (90%) non-baryonic DM component. We ran three realizations of each model and averaged the results. Figure 1 shows a time-sequence of the projected galaxy distribution in co-moving coordinates for the model with DM. The co-moving box size here corresponds to $2R$, that is, to $6.7 R_s$. Visually, the smooth 'bubbly' structure which is apparent in the initial conditions tends to smear out with time because of gravitational clustering both within the individual shells and due to the interaction between different shells. The parts of the shells that overlap tend to become locally bound to each other: they form regions of enhanced clustering which visually resemble the observed elongated superclusters of galaxies and clusters. It seems that the superclusters mainly form in the regions of shell overlap.

These projected views are not in a form directly comparable to the 'slice' representation of de Lapparent *et al.*: the depth of each of our projections is typically $\sim 50 h^{-1}$ Mpc (for $R_s = 10 h^{-1}$ Mpc), while their slice corresponds to only $\sim 10 h^{-1}$ Mpc at a distance of $100 h^{-1}$ Mpc (where the big 'bubble' is observed). Similar slices of our simulations do show certain 'bubbly' structures but they are rather 'frothy' as the bubbles break down into smaller groups and clumps at late times. It would therefore be naive to expect round smooth bubbles to be a typical consequence of the explosion scenario. Indeed, the equivalent slices of a larger volume towards the Perseus-Pisces supercluster show a rather clumpy structure on large scales with no smooth bubbles¹⁴.

Figure 2 shows the time evolution of the galaxy correlation function $\xi_{gal}(r)$ in the model with no DM (averaged over three

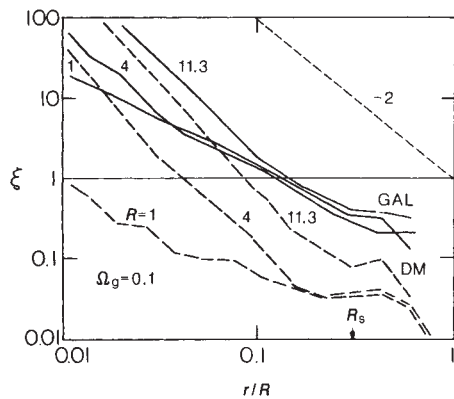


Fig. 3 Co-moving evolution of the averaged galaxy-galaxy correlation function (solid lines) against that of the dark matter (broken lines) in the scenario with $\Omega_g = 0.1$.

simulations). The quantity $1 + \xi_{\text{gal}}(r)$ is determined by counting pairs with separation in the separation interval $(r, r + \Delta r)$ and dividing this by the corresponding number of pairs in a Poisson distribution with the same number of points in the same volume (for example, ref. 23). The correlation function at the initial time looks like $\xi_{\text{gal}}(r) \propto r^{-1}$ for values of r larger than the mean galactic separation but smaller than the shell size; the correlation length (where $\xi_{\text{gal}} = 1$) is $r_c \approx 0.1 R$. This is just what is expected²³ for a distribution of points on two-dimensional surfaces with characteristic scale and separations of a few r_c . The correlation function then steepens in time due to gravitational clustering on progressively growing comoving scales. It attains a logarithmic slope of roughly -1.8 , as observed²⁴, at an expansion factor between $R = 4$ and $R = 8$, and it becomes even steeper afterwards. The scenario would therefore require galaxy formation at $z \approx 5$. The correlation length at this epoch, $r_c \approx 0.15 R$, would correspond to $5 h^{-1}$ Mpc, as observed²⁴, if $R_s = 0.3 R$ corresponds to $10 h^{-1}$ Mpc, so there is a rough agreement between the scale of clustering expected in the explosion model and that observed.

The model with $\Omega_g = 0.1$ is probably more realistic in view of the primordial nucleosynthesis constraints²⁰. Figure 3 shows the co-moving evolution of ξ_{gal} in this case against the evolution of the DM correlation function ξ_{DM} . As before, ξ_{gal} steepens with time but it does so at a slower rate, reaching $\xi_{\text{gal}} \propto r^{-1.8}$ only at $R = 11$ (that is, galaxy formation occurs at $z = 10$). The DM has a roughly Poisson distribution initially and so ξ_{DM} is initially very small. This introduces an initial 'bias' in the (clumped)

galaxy distribution against the (smooth) DM distribution. However, the DM fluctuations grow for the following reasons: (i) the shell interiors are slowly evacuated dynamically due to the underdensity introduced by the gas within them having been swept out; (ii) the DM accretes onto the galaxies; and (iii) the initial Poissonian component of the DM fluctuations grows self-gravitationally. The white noise fluctuations introduced by the last effect resemble the cold DM fluctuations on the scales of relevance here; they become the dominant component after $R = 11$. When the galaxy and DM distributions eventually coincide, the 'bias' between them disappears. The present correlation length is again $\sim 5 h^{-1}$ Mpc, in pleasant agreement with observations.

Apart from the slope of ξ_{gal} , an interesting constraint comes from the degree of bias, expressed for example by the ratio $\xi_{\text{gal}}/\xi_{\text{DM}}$ at r_c ; this ratio is a decreasing function of time which approaches unity at late epochs. Motivated by a desire to reconcile the elegant $\Omega = 1$ cosmological model (predicted by inflation) with the dynamics of the Local Supercluster and the cosmic virial analysis of galaxy pairs, this ratio should today be $\xi_{\text{gal}}/\xi_{\text{DM}} \approx 5$ (ref. 25 and A. Dekel, J. Einasto and M. J. Rees, in preparation). It is evident from Fig. 3 that this degree of bias indeed occurs at $R = 11$, indicating a constraint on the epoch of galaxy formation similar to that obtained on the basis of the slope of ξ_{gal} .

Finally, we make an attempt to quantify the voids, using the distribution of distances to the nearest galaxy from each point of a cubic grid²⁶. We choose the grid spacing to be the mean galaxy separation, and express the distances, d , in terms of the same mean separation. Figure 4 compares the averaged histogram obtained in our simulations with the one obtained by Ryder and Turner²⁷ for an observed volume-limited subsample of the CfA redshift survey. The latter corresponds to the subsample limited to absolute magnitudes $M \leq -19$, which contains 489 galaxies out to a distance $r = 25 h^{-1}$ Mpc. The histogram corresponding to the other subsample analysed by Ryder and Turner ($M \leq -21.5$, 254 galaxies, $r = 76 h^{-1}$ Mpc), which is not shown, is in general shifted towards smaller relative separations d . The void distribution in the simulations clearly evolves in time towards larger average voids. At the epoch of galaxy formation, the theoretical voids tend to be smaller than those observed but they match each other at $R \approx 8$ in the $\Omega_g = 1$ model and at $R > 11$ in the $\Omega_g = 0.1$ model. The maximum value of d , which is an indication for the radius of the largest void, is 2.4 in the simulations; this corresponds to $13 h^{-1}$ Mpc (for $R_s = 10 h^{-1}$ Mpc), compared with $d = 3.4$ in the observed sample ($M \leq -19$), which corresponds to $10.4 h^{-1}$ Mpc. (This statistic, however, should be considered with caution because it may

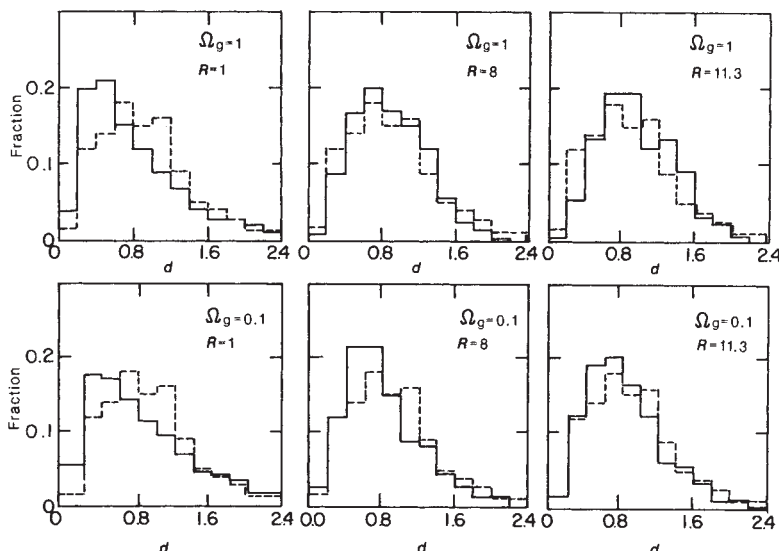


Fig. 4 Evolution (left to right) of the distribution of distances of nearest galaxies from uniform grid points in the two simulations (solid lines) compared with the results from the observed sample (broken line).

depend on the number density of galaxies in a non-trivial way, and it might be affected by edge effects.)

These results, combined with the results based on the correlation function and the visual appearance of the clustering pattern, seem to confirm the view that the large-scale distribution of galaxies in the explosion model evolves gravitationally to a distribution which roughly resembles the observed distribution at a certain time after the epoch of galaxy formation. The inferred epoch of galaxy formation is $z \approx 5$ for a baryonic Universe ($\Omega_g = 1$) and $z \approx 10$ for a Universe dominated by non-baryonic DM ($\Omega_g = 0.1$). Despite the statements sometimes made in the literature^{14,28}, our preliminary results indicate that it is not impossible to generate the scale of structure observed without appeal to other sources of large-scale fluctuations. Possible exceptions might be the excess clustering of rich Abell clusters²⁹ on scales $100 h^{-1}$ Mpc and the existence of 'voids' with diameters exceeding $50 h^{-1}$ Mpc. However, such features require a non-trivial explanation in all cosmogonic models currently under consideration. More extensive studies of the issues discussed here are currently in progress¹⁰ (see D. Weinberg, A. Dekel and J. P. Ostriker, in preparation).

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1. Dekel, A. & Rees, M. J. *Nature* (submitted).
2. Ostriker, J. P. & Cowie, L. L. *Astrophys. J.* **243**, L127-L131 (1981).
3. Ikeuchi, S. *Publ. astr. Soc. Japan* **33**, 211-222 (1981).
4. Carr, B. J. & Ikeuchi, S. *M.N.R.A.S.* **213**, 497-518 (1985).
5. Ikeuchi, S., Tomisaka, K. & Ostriker, J. P. *Astrophys. J.* **265**, 583-596 (1983).
6. Vishniac, E. T., Ostriker, J. P. & Bertschinger, E. *Astrophys. J.* **291**, 399-416 (1985).
7. Wandel, A. *Astrophys. J.* **294**, 385-396 (1985).
8. Bertschinger, E. *Astrophys. J.* **295**, 1-13 (1985).
9. Bertschinger, E. *Astrophys. J.* **268**, 17-29 (1983).
10. Allen, A. J. & Carr, B. J. (preprint).
11. Oort, J. H. A. *Rev. Astr. Astrophys.* **21**, 373-428 (1983).
12. Tully, R. B. *Astrophys. J.* **257**, 389-422 (1982).
13. Giovanelli, R., Haynes, M. P. & Chincarini, G. *Astrophys. J.* **300**, 77-92 (1986).
14. de Lapparent, V., Geller, M. & Huchra, J. *Astrophys. J.* **302**, L1-L5 (1986).
15. Giovanelli, R. & Haynes, M., in *Galaxy Distances and Deviations from Universal Expansion* (eds B. F. Madore & R. B. Tully, Dordrecht, Reidel, 1986).
16. Bean, A. J., Efstathiou, G., Ellis, R., Peterson, B. A. & Shanks, T. *Mon. Not. R. astr. Soc.* **205**, 605-624 (1983).
17. Batiski, D. J. & Burns, J. O. *Astrophys. J.* **299**, 5-14 (1985).
18. Szalay, A. S. & Koo, D. C. in *Galaxy Distances and Deviations from Universal Expansion* (eds B. F. Madore & R. B. Tully, Dordrecht, Reidel, 1986).
19. Kirshner, R., Oemler, A., Schechter, P. & Schechtman, S. A. *Astrophys. J.* **248**, L57-L60 (1981).
20. Boesgaard, A. & Steigman, G. A. *Rev. Astr. Astrophys.* **23**, 319-378 (1985).
21. Carr, B. J. *Nucl. Phys. B* **252**, 81-111 (1985).
22. Aarseth, S. J. in *Methods of Computational Physics* (New York, Academic Press) (eds J. U. Brackbill and B. I. Cohen, 1984).
23. Dekel, A. & Aarseth, S. J. *Astrophys. J.* **283**, 1-23 (1984).
24. Davis, M. & Peebles, P. J. E. *Astrophys. J.* **267**, 465-482 (1983).
25. Dekel, A. *Comments Astrophys.* (in the press).
26. Aarseth, S. J. & Saslaw, W. C. *Astrophys. J.* **258**, L7-L10 (1982).
27. Ryden, B. S. & Turner, E. L. *Astrophys. J.* **287**, L59-L63 (1984).
28. Peebles, P. J. E. *Nature* **321**, 27-32 (1986).
29. Bahcall, N. & Soneira, R. *Astrophys. J.* **270**, 20-38 (1983).

Cubic ice from liquid water

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Hexagonal ice (ice Ih) is the only form of ice that is known to occur naturally on the Earth. Recently it has been suggested that small droplets of water in the upper atmosphere may often freeze first to cubic ice (ice Ic), which is metastable relative to ice Ih. The evidence is Scheiner's halo, a rare halo that occurs at $\sim 28^\circ$ from the Sun or the Moon¹⁻³, and the observation that dendritic snowflakes often have their *c*-axes at about 70° to one another^{4,5}. Here we report the formation of cubic ice from liquid water at ≤ 200 K, using a method for vitrifying pure liquid water, namely

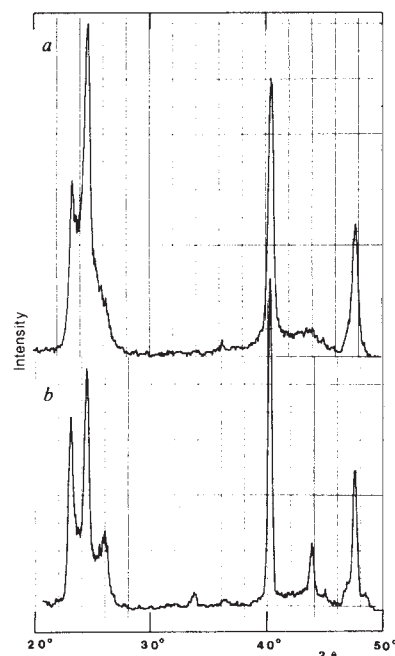


Fig. 1 X-ray diffractograms (CuK α , 100 K) of water droplets quenched (a) at 190 K and (b) at 200 K. The cubic ice samples were prepared as described in earlier reports for the vitrification of liquid water^{6,7}, using only higher cryoplate temperatures. Aqueous aerosol droplets, made by an ultrasonic nebulizer with $\leq 3 \mu\text{m}$ diameter, were transferred through a small opening, with nitrogen as carrier gas, into a high vacuum system, accelerated by supersonic flow and deposited on an X-ray sample holder acting as cryoplate. The sample holder was attached on a Displex 202 cryotip (Air Products) and the temperature regulated with the APD-E system. To obtain high cooling rate, the carrier gas has to be pumped off or condensed out. We used a second high-capacity Displex (model CS-208L) as cryopump. Deposition time was 30 min. Cryoplate temperatures were accurate at least to ± 1 K. Both sample holder and deposited sample were transferred to the pre-cooled low-temperature X-ray camera under liquid nitrogen.

rapid quenching of aqueous aerosol droplets $\sim 3 \mu\text{m}$ in diameter on a cryoplate^{6,7}. Cubic ice made from liquid water transforms much more slowly to the stable form than sample prepared by deposition of the vapour. Speculations about naturally occurring ice Ic (refs 1-5) are supported by our results.

This seems to be the first unambiguous report of the direct formation of cubic ice from bulk samples of pure liquid water. Earlier reports of cubic ice formation as a by-product of the vitrification of liquid water^{8,9} were hampered by the fact that the low deposition temperatures (< 100 K) in these experiments did not differentiate between formation from the amorphous phase and formation directly from the liquid. Likewise, the recently reported formation of cubic ice in a porous silica network¹⁰ at 260 K is probably a result of geometric constraints of the small pores and/or of changes in the bulk properties of the liquid enclosed in the pores.

Figure 1 shows the X-ray diffractograms of water droplets quenched rapidly (a) at 190 K and (b) 200 K. The pattern in Fig. 1b indicates a mixture of ice Ic and Ih. The reflections in Fig. 1a are mainly from cubic ice. Only the 100 reflection of ice Ih (at $2\theta = 22.8^\circ$ for CuK α) is observable as a sharp peak; the other ice Ih reflections are either very broad or absent. Similar patterns of ice Ic made from the high-pressure forms of ice have recently been analysed in terms of stacking faults¹¹.

The cubic ice samples were prepared using conditions where cubic ice made by deposition of water vapour is known to transform to ice Ih¹². This demonstrates best the enhanced thermal stability of ice Ic when made from the liquid. We investigated the conversion to ice Ih in the X-ray diffractometer, using the growth of the 103 reflection (at $2\theta = 43.7^\circ$) as an

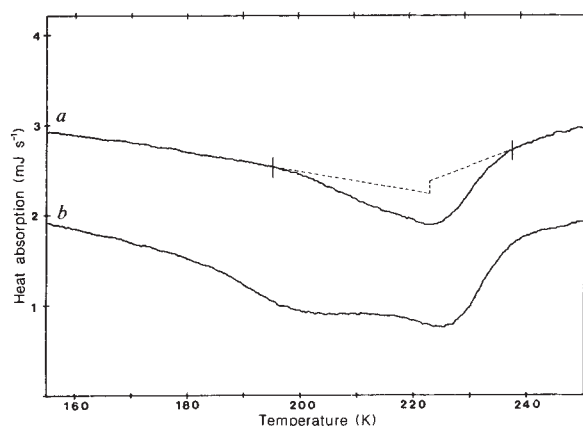


Fig. 2 Thermograms of cubic ice samples prepared by quenching of water droplets (a) at 190 K, and (b) at 170 K. The samples were investigated on a computerized Perkin-Elmer DSC-4 instrument. Heating rate was 10 K min⁻¹. Broken lines in a indicate the baseline chosen for the enthalpy estimate; vertical bars, the limits of integration. This type of baseline is one of the standard methods for approximating baselines with shift by extending both pre-event and post-event baselines until they connect with a line dropped vertically from the peak temperature point¹⁵. The mass of the water samples was determined from the heat of melting.

indicator. After 30 min at 230 K, only about 70% of ice Ic had transformed, and even at 240 K similar times were necessary to obtain the final intensity of the 103 reflection.

We have in addition investigated the phase transition by differential scanning calorimetry (DSC). Figure 2a shows the thermogram of a cubic ice sample prepared as for Fig. 1a, by rapid quenching at 190 K. The broad exotherm with a peak maximum at about 223 K is due to the ice Ic → Ih phase transition. Identical curves have been obtained from samples of vitrified liquid water prepared by quenching at 77 K. A baseline shift makes an exact evaluation of the enthalpy of transition problematic. An estimate of -56 J mol⁻¹ was obtained for a baseline shown in Fig. 2a with broken lines¹³. This estimate compares well with the recently determined value of -50.5 J mol⁻¹ obtained on cubic ice with small surface area¹⁴.

In cubic ice samples prepared by quenching at 170 K, we observed an additional weak exotherm peaking between 190 and 200 K, as shown in Fig. 2b. It disappears after annealing at 190 K for 5 min, giving the same thermogram as in Fig. 2a. We ascertained by X-ray diffraction that these annealing conditions produced no hexagonal ice. However, we observed in samples quenched at 170 K that the 100 reflection of ice Ih is present only as a shoulder, and that it sharpens to a separate peak when warmed at 200 K, giving the same pattern as in Fig. 1a. Both effects occur in the same temperature region and might be related.

The differences in thermal stability between cubic ice made from the vapour and the liquid are probably the result of differences in surface areas. For nucleation-controlled solid-state transformations the conversion rate is strongly size-dependent¹⁵, powders transforming more rapidly than larger crystals. It has been shown recently that ice Ic made from vapour has a large surface area¹⁴. For cubic ice made from water droplets, a much smaller surface area is to be expected and surface-induced nucleation should be slower. Cubic ice crystals made from water droplets in the upper atmosphere may be even more stable than our compact samples because they are isolated from one another.

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1. Whalley, E. *Science* **211**, 389-390 (1981).
2. Whalley, E. *J. phys. Chem.* **87**, 4174-4179 (1983).
3. Whalley, E. & McLaurin, G. E. *J. opt. Soc. Am. A*, **1**, 1166-1170 (1984).

4. Kobayashi, T., Furukawa, Y. & Takahashi, T. *J. Cryst. Growth* **35**, 262-268 (1976).
5. Takahashi, T. & Kobayashi, T. *J. Cryst. Growth* **64**, 593-603 (1983).
6. Mayer, E. *J. appl. Phys.* **58**, 663-667 (1985).
7. Mayer, E. *J. phys. Chem.* **89**, 3474-3477 (1985).
8. Brüggeller, P. & Mayer, E. *Nature* **288**, 569-571 (1980).
9. Dubochet, J. & Lepault, J. *J. de Physique C7*, 85-94 (1984).
10. Steytler, D. C., Dore, J. C. & Wright, C. J. *J. phys. Chem.* **87**, 2458-2459 (1983).
11. Kuhs, W. F., Bliss, D. V. & Finney, J. L. *VII Symposium on the Physics and Chemistry of Ice* (Grenoble, 1986).
12. Hobbs, P. V. in *Ice Physics*, 58 (Clarendon, Oxford 1974).
13. Brennan, W. P., Miller, B. & Whitwell, J. C. *Ind. Eng. Chem. Fund.* **8**, 314-318 (1969).
14. Handa, Y. P., Klug, D. D. & Whalley, E. *J. chem. Phys.* **84**, 7009-7010 (1986).
15. Cardew, P. T., Davey, R. J. & Ruddick, A. J. *JCS Faraday Trans II*, **80**, 659-668 (1984).

Pesticides in fog

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The discovery of the very acidic nature of fog and clouds^{1,2} has created much interest in sampling, analysing, and elucidating the chemistry of fog, principally because an understanding of the chemical transformations leading to acid fog may provide important clues to the origin of acid rain. Recently, the knowledge of the chemistry of fog has expanded to include carbonyl compounds³, volatile organic acids⁴, and alkyl sulphonates^{5,6}. We have discovered that a variety of pesticides and their toxic alteration products are present in fog, and that they occasionally reach high concentrations relative to reported rainwater concentrations. In our experiments, we were able to measure the air-water distribution coefficients of pesticides between the liquid fog and the interstitial gas phase. These measurements reveal that some chemicals are enriched several thousandfold in the suspended liquid fog droplets compared to equilibrium distributions expected from Henry's Law coefficients for pure aqueous solutions.

Litre-sized samples of fog liquid were collected using a high-volume, rotating stainless steel-screen collector, described elsewhere⁷. We also sampled interstitial gases and unactivated aerosol particles using a high-volume dichotomous sampler. We modified the original sampler⁸ to achieve a droplet cutoff diameter of about 8 µm. Fog droplets were thus removed from the air, which was then passed first through a glass-fibre (GF) filter to trap particles, and then through a bed of porous polyurethane foam to trap vapours of pesticides and other organics⁹. The glass-fibre filter and polyurethane foam trap were extracted in a Soxhlet apparatus for 6 h using 1:1 hexane/acetone. Fog liquid was filtered through a glass-fibre filter, and filter-retained pesticides were recovered by a 12-h Soxhlet extraction with dichloromethane. The filtered fog water was extracted with dichloromethane using an established multi-residue procedure¹⁰. Concentrated sample extracts were split into four fractions of increasing polarity by silica gel HPLC and a hexane- to-methyl *t*-butyl ether gradient¹¹. The samples were then analysed by element-selective gas chromatography, or by gas chromatography-mass spectrometry.

Fog samples were collected at the US Department of Agriculture's Beltsville Agricultural Research Center (BARC), located at Beltsville, Maryland, a suburban residential area about 15 km north-east of Washington, DC, and at various locations in the San Joaquin Valley in California. We will discuss the following four sampling events: 7 May 1984, in an area of fields and orchards on the BARC-West campus that is used for various types of experimental work; 13 January 1985 (a.m.), in the southern San Joaquin cotton-growing area near Corcoran, California; 13 January 1985 (p.m.), at the Kearney Agricultural Research Center at Parlier, California (near Fresno) in an area

Table 1 Distribution of pesticides, their alteration products, and tributylphosphate between fogwater and interstitial air

Chemical concentration, ng l ⁻¹			Distribution ratio (×10 ⁶) air-water		Enrichment factor	
		fog water	Air (×10 ³)	measured (D)	literature (H)	EF
-Organophosphorus insecticides-						
Diazinon	P	16,600	2.2	0.12	60	500
	C	11,800	4.2	0.36		170
	L	22,000	5.3	0.24		249
	B	140	8.6	60		1
Parathion	P	12,400	3.2	0.25	9.5	38
	C	5,800	0.88	0.15		63
	L	51,400	7.9	0.15		63
Chlorpyrifos	P	1,020	3.3	3.2	500	156
	C	320	0.6	1.9		260
	L	6,500	14.7	2.3		217
Methidathion	P	840	<0.03	<0.04	0.07	>1.8
	C	570	<0.01	<0.02		>3.5
	L	15,500	<0.6	<0.04		>1.8
Malathion	P	70	<0.03	<0.4	2.4	>6
	C	110	0.02	0.18		13
	L	350				
	B	2,740	5.2	1.9		1.3
Methyl parathion	B	1,210	7.3	6.0	4.4	0.7
-Oxygen analogues-						
Parathion OA	P	9,000	0.21	0.023	0.25	11
	C	950	0.066	0.07		3.6
	L	184,000	0.25	0.001		250
Methidathion OA	P	120	0.03	<0.25	—	—
	L	8,200				
Chlorpyrifos OA	P	170	<0.03	<0.18	—	—
	L	800				
Diazinon OA	P	190	<0.03	<0.16	—	—
-Cotton defoliant-						
DEF	P	250	<0.03	<0.12	320	>2,700
	C	800	0.14	0.18		1,800
-Herbicides-						
Atrazine	P	270	<0.2	<0.7	0.2	—
	C	320	<0.16	<0.4		—
	L	700	—	—		—
	B	820	3.4	4.1		0.05
Simazine	P	390	<0.1	<0.3	0.025	—
	C	110	<0.06	<0.5		—
	L	1,200	—	—		—
	B	45	<0.04	<0.8		—
Pendimethalin	P	1,370	0.64	0.47	1,500	3,200
	C	3,620	3.6	1.0		1,500
Alachlor	B	1,450	<0.4	<0.3	1.3	>4
Metolachlor	B	1,960	<0.4	<0.2	0.37	>2
-Flame-retardant plasticizer-						
Tributyl-phosphate	B	1,090	0.68	0.62	26	42

EF (=ratio of literature to measured distribution ratio) reflects aqueous-phase enrichment. P=Parlier, CA; C=Corcoran, CA; L=Lodi, CA.; B=Beltsville, MD.

dominated by fruit and citrus orchards; and 19 January 1985, in a dairy and grape-growing area near Lodi, California.

We found aerosol particles and non-pesticide extractable organic matter in all the fog samples. The Beltsville, Parlier, and Corcoran samples were nearly clear, but the Lodi sample was heavily loaded with particulate matter and was opaque. At most, only traces of pesticide were associated with the particles. Filtered solutions were pale yellow, with a large portion of the colour being dichloromethane-extractable organic matter. All fog liquids foamed to a certain extent, most noticeably the Lodi sample. The Beltsville sample was very acidic ($pH=2.42$), the major acidic species being sulphate (SO_4^{2-}/NO_3^-) ≈ 2 . The California samples were much less acidic (pH range, 5.1–7.0). The inorganic chemistry of Central Valley fogs has been previously elucidated¹².

Table 1 gives the 16 pesticides and their alteration products

identified in fog. As a class, the organophosphorus insecticides (diazinon, parathion, chlorpyrifos, methidathion, malathion, methyl parathion) and their oxygen analogues (oxons) were most numerous. We also found several classes of herbicides: *s*-triazines (atrazine, simazine), dinitroaniline (pendimethalin), and chloracetanilides (alachlor, metolachlor). The California fog samples yielded a greater variety and higher concentration of pesticides than Beltsville, a result attributable to the larger quantities and more diverse use of pesticides in San Joaquin Valley agriculture. Table 1 shows that the organophosphorus insecticides frequently exceeded $10 \mu g l^{-1}$ in fog, which is two to three orders of magnitude greater than commonly reported for pesticides and other neutral organics in rain from other locations^{13–15}. Elevated levels of diazinon and parathion in Parlier fog may reflect local use as wintertime dormant sprays on fruit trees. This does not explain the $50 \mu g l^{-1}$ parathion

found in Lodi fog, since this sample was collected in a dairy pasture area where wintertime dormant spraying is not expected.

California fog contained a number of the toxic oxygen analogues (OAs) of organophosphorus insecticides. The OA of parathion, paraoxon, was most abundant, but we also found chlorpyrifos OA, methidathion OA, and diazinon OA. Their source is uncertain. It is known that OAs form in gas-phase reactions of the parent insecticides with ozone^{16,17}, and also occur in high concentrations in dry surface dust^{17,18}, but because high oxidant levels are possible in fog, *in situ* conversion within fog droplets cannot be ruled out. OAs are potent choline esterase inhibitors and are responsible for the toxic effects of the organophosphorus insecticides¹⁹. This is of concern because the highest 'pesticide' concentration we measured was $184 \mu\text{g l}^{-1}$ of paraoxon in Lodi fog. The laboratory-measured choline esterase inhibition of Lodi fog extracts matched that of equivalent concentrations of pure paraoxon standard.

It is commonly assumed that the distribution of low solubility organics between water and air is described by Henry's Law, and that the air-to-water distribution coefficient is given by the ratio of vapour density to solubility of the pure organic chemical¹³⁻¹⁵. We used vapour density and solubility to calculate H as a dimensionless distribution ratio, $(\text{ng l}^{-1}) \text{ vapour}/(\text{ng l}^{-1}) \text{ dissolved}$. For those pesticides identified in fog, this calculated distribution ratio, H , ranges over five orders of magnitude: from 2.5×10^{-8} (simazine) to 1.5×10^{-3} (pendimethalin). Compounds with smaller H have a greater tendency to associate with the aqueous phase. A number of compounds with $H < 10^{-6}$ were found in fog even though their interstitial air concentrations were immeasurably low.

H was not a good quantitative estimate of the distribution of pesticides between fog droplets and air in our samples. The measured air-water distribution ratio, D , was frequently very much less than H (Table 1), especially for the California samples. $D < H$ implies an aqueous-phase enrichment: more pesticide vapour dissolves in the fog droplet than would dissolve in an ideal solution at equilibrium. The extent of this aqueous-phase enrichment is revealed by the enrichment factors ($\text{EF} = H/D$) shown in Table 1, which were large in several cases. We measured several thousandfold enrichments for DEF and pendimethalin. The reason for these high enrichments is not known, but the nature of the enrichment factors and the different conditions under which the various samples were collected rule out temperature effects and sampling artifacts.

It is remarkable how similar the EF values were for each pesticide in the three California samples, even though they were obtained under different conditions at widely separated locations. For these samples there was a relationship between EF and H : aqueous-phase enrichment increased as H increased, that is, for those pesticides which were more volatile and/or less soluble. This function, shown in Fig. 1, indicated that aqueous-phase enrichment was important in the California samples when $H > 10^{-7}$, and increased smoothly, approximately as $H^{2/3}$, over four orders of magnitude ($\log \text{EF} = 0.66 H + 5.02$; $r^2 = 0.88$). It may be that pesticides with $H < 10^{-7}$ exhibit no aqueous-phase enrichment because of their high inherent affinity for the aqueous phase. The very high paraoxon concentration in the Lodi fog appears to be anomalous.

In addition to the data presented in Table 1, samples of eight fog events at other locations in California's Central Valley have been analysed for diazinon and parathion. For these samples, diazinon enrichment ranged from 7 to 1,100, and averaged 380; parathion enrichment ranged from 3 to 59 and averaged 20. These enrichments are consistent with those reported for Corcoran, Parlier, and Lodi in Table 1, and suggest that aqueous-phase enrichment occurs quite regularly in Central Valley fogs.

Enrichment factors for the Beltsville sample, also plotted in Fig. 1, provided an interesting contrast. Of the chemicals found in the Beltsville sample, three (alachlor, metolachlor, and tributylphosphate) were enriched in a manner similar to the

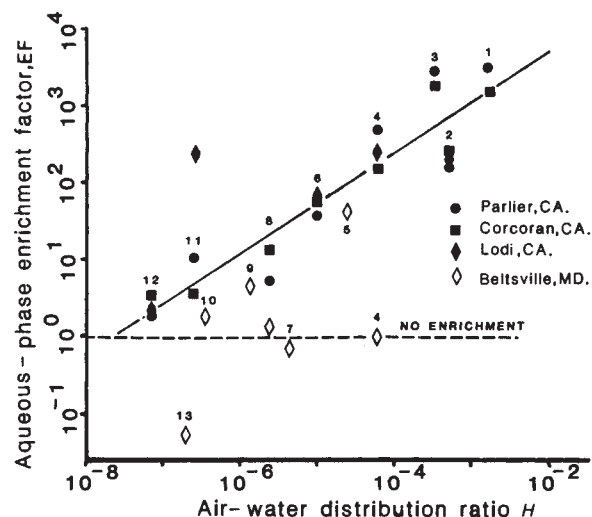


Fig. 1 Enhanced uptake of pesticides from interstitial air into fog droplets. The enrichment factor (EF, from Table 1) is the ratio of literature to measured air/water distribution ratios. 1, Pendimethalin; 2, chlorpyrifos; 3, DEF; 4, diazinon; 5, tributylphosphate; 6, parathion; 7, methyl parathion; 8, malathion; 9, alachlor; 10, metolachlor; 11, paraoxon; 12, methidathion; 13, atrazine.

California samples; three (diazinon, methyl parathion and malathion, all organophosphorus insecticides) were not enriched; and one (atrazine) had 20-fold less in the aqueous phase than expected. The grouping of classes of pesticides in this manner suggests that the phenomenon controlling enrichment results from very selective molecular or functional group interactions.

Enrichment into the fog droplet was more pronounced for hydrophobic pesticides than hydrophilic ones (Fig. 1). One hypothesis to explain enrichment is that fog droplets contained solutes, such as dissolved or perhaps colloidal organic material, which increased the solubility of hydrophobic pesticides, thereby shifting the equilibrium to the solution phase. A plot similar to Fig. 1 of California-sample EF against pesticide water solubility showed that aqueous-phase enrichment generally increased with decreasing solubility, but a much poorer linear relationship resulted ($\log \text{EF} = -0.75 \log S + 2.88$; $r^2 = 0.75$). The data therefore indicate that solubility and vapour pressure are both important to the enrichment process.

Surface-active material has previously been reported in fog²⁰. We know that such non-pesticidal organic matter was present in our samples, as shown by their foamy, soapy appearance. Although we have no experimental verification, it seems a reasonable conjecture that surface-active material might have been present in amounts sufficient to produce an organic film on the droplet surfaces²⁰. A second hypothesis, then, is that surface-active organic matter present at the air-water interface acted to enhance the uptake of pesticides into the aqueous phase. This interpretation is consistent with the chemical-class specific enrichments found for the Beltsville fog. The very low pH of the Beltsville fog could have changed the character and interactions of fog-droplet surfactants.

Whatever the cause, it is clear there has been a shift in equilibrium, and that fog droplets are dramatically different from ideal solutions. The data show an enhanced uptake of volatile, hydrophobic organics, and suggest that non-pesticidal organics may be influencing the air-water distribution. Dissolved and colloidal organics are known to affect the solubility and air-water distribution of hydrophobic pollutants in this manner in laboratory systems²¹.

Our results for the air-water distribution in fog might have implications for pesticides in other aquatic media, and particularly for other environmentally-important organics in fog. The high deposition velocity of fog droplets might represent a

means of exposure for vegetation^{22,23}. When fog forms, exposed plant surfaces become saturated with fog liquid for the duration of the fog event, and some organic residues may become more concentrated on the surface as moisture evaporates during fog dissipation. Also, the regional distribution of organics in fog indicated in our San Joaquin Valley samples may signal another mechanism whereby chemicals capable of entering the atmosphere may move to surfaces some distance from the target. The

environmental significance of fog as a means of concentrating, moving, and depositing organic atmospheric pollutants, such as pesticides, warrants further attention.

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1. Falconer, R. E. & Falconer, P. D. *J. geophys. Res.* **85**, 7465-7470 (1980).
2. Munger, J. W., Jacob, D. J., Waldman, J. M. & Hoffmann, M. R. *J. geophys. Res.* **88**, 5109-5121 (1983).
3. Grosjean, D. & Wright, B. *Atmos. Environ.* **17**, 2093-2096 (1983).
4. Kawamura, K. & Kaplan, I. R. *Analyt. Chem.* **56**, 1616-1620 (1984).
5. Munger, J. W., Jacob, D. J. & Hoffmann, M. R. *J. atmos. Chem.* **1**, 335-350 (1984).
6. Munger, J. W., Tiller, C. & Hoffmann, M. R. *Science* **231**, 247-249 (1986).
7. Glotfelty, D. E., Seiber, J. N. & Liljedahl, L. A. *Proc. Symp. Measurement of Toxic Air Pollut.* (ed. Raleigh, N. C.) 168-175 (Air Pollut. Control Assoc/EPA).
8. Solomon, P. A., Moyers, J. L. & Fletcher, R. A. *Aerosol Sci. Technol.* **2**, 455-464 (1983).
9. Keller, C. D. & Bidleman, T. F. *Atmos. Environ.* **18**, 837-845 (1984).
10. Thompson, J. F., Reid, S. J. & Kantor, E. J. *Arch. Environ. Contam. Toxicol.* **6**, 143-157 (1977).
11. Wehner, T. A., Woodrow, J. E., Kim, Y.-H. & Seiber, J. E. in *Identification and Analysis of Organic Pollutants in Air*, 273-290 (ed. Keith, L. H., Butterworth, Boston, 1984).
12. Jacob, D. J., Waldman, J. M., Munger, J. W. & Hoffmann, M. R. *Tellus* **36B**, 272-285 (1984).
13. Eisenreich, J. S., Looney, B. B. & Thornton, J. D. *Environ. Sci. Technol.* **15**, 30-38 (1981).
14. Harder, H. W., Christensen, E., Matthews, J. R. & Bidleman, T. F. *Estuaries* **3**, 142-147 (1980).
15. Ligoicki, M. P., Leuenberger, C. & Pankow, J. F. *Atmos. Environ.* **19**, 1609-1617 (1985).
16. Woodrow, J. E., Crosby, D. G., Mast, T., Moilanen, K. W. & Seiber, J. N., *J. agric. Food. Chem.* **26**, 1312-1316 (1978).
17. Woodrow, J. E. *et al. Arch. environ. Contam. Tox.* **6**, 175-191 (1977).
18. Spencer, W. F., Shoap, T. D. & Spear, R. C. *J. agric. Food Chem.* **28**, 1295-1300 (1980).
19. Spear, R. C., Popendorf, W. J., Spencer, W. F. & Milby, T. H. *J. occup. Med.* **19**, 411 (1977).
20. Gill, P. S., Graedel, T. E. & Weschler, C. J. *Rev. Geophys. Space Phys.* **21**, 903-920 (1983).
21. Hassett, J. P. & Milicic, E. *Environ. Sci. Technol.* **19**, 638-643 (1985).
22. Waldman, J. M., Munger, J. W., Jacob, D. J. & Hoffmann, M. R. *Tellus* **37B**, 91-108 (1985).
23. Roach, W. T., Brown, R., Caughey, S. J., Garland, J. A. & Readings, C. J. *Q. Jl R. met. Soc.* **102**, 313-333 (1976).

Upper mantle origin for Harding County well gases

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Short-lived radioisotopes such as ¹²⁹I and ²⁴⁴Pu represent extremely powerful geochemical tools for tracing the early history of the Earth. Terrestrial ¹²⁹Xe anomalies were first found in CO₂-rich well gases from Harding County, New Mexico, and then in gases from mid-ocean-ridge basalts (MORB). Here I show that noble gases and CO₂ from Harding County are isotopically indistinguishable from such gases in fresh MORB glasses and that both probably have the same source, namely the upper mantle.

In the past 25 years, CO₂ well gases in general¹ and CO₂-rich well gases from Harding County, New Mexico, in particular, have been extensively investigated for noble gases by several authors. In 1963, Butler *et al.*² first found a small, but significant ¹²⁹Xe excess (~2%) and fissionogenic Xe excesses in CO₂-rich well gases from the Bueyeros field in Harding County (Mitchell no. 7). They interpreted such excesses to be formed by ¹²⁹I decay and by spontaneous fission of ²³⁸U or ²⁴⁴Pu and supposed them "to reflect the isotopic composition of deep seated terrestrial gas". Two years later, Wasserburg and Mazor³ reported a much higher ¹²⁹Xe excess (8.3%) and similar fission Xe excesses in an aliquot of Mitchell no. 7. The ⁴⁰Ar/³⁶Ar ratios, however, range between 3,000 and 24,000 in Harding County well gases (Mitchell nos 4, 7 and 12).

Boulos and Manuel⁴ in 1971 found still higher ¹²⁹Xe excess (9.7%) and fissionogenic Xe excesses. In 1975, Hennecke and Manuel⁵ published noble-gas data on three further gas samples

from Harding County, showing Xe excesses relative to air similar to those of Boulos & Manuel, ⁴⁰Ar/³⁶Ar ratios > 7,000, and enhanced ²⁰Ne/²²Ne and ²¹Ne/²²Ne ratios. The most comprehensive noble-gas study on CO₂-rich well gases from the Bueyeros field, however, was carried out by Phinney *et al.*⁶ in 1978. They measured the concentrations of all noble gases and the compositions of He to Xe, providing strong evidence of a primordial component in Harding County CO₂-rich well gases. As stated by Phinney *et al.*, excesses of ³He and ¹²⁹Xe clearly show that a mantle component is present, but without a knowledge of the exact isotope signature of upper mantle noble gases, it was impossible at that time to evaluate the importance of this primitive component. In fact, CO₂-rich well gases could be a mixture of different reservoirs: atmosphere, continental crust, upper mantle.

We claim that Harding County CO₂-rich well gases represent pure noble gases derived from the upper mantle and are, except for the He component, not contaminated by gases derived from the continental crust.

In the next section, the discussion focuses on the isotope signatures of each noble gas and of carbon in CO₂.

Phinney *et al.*⁶ reported a ⁴He/³He ratio of 2.28 × 10⁵. Such a value is intermediate to that of typical MORB and atmosphere or continental crust (Table 1). Because of the low abundance of He in air, atmospheric contamination is not a likely explanation for this value, because together with atmospheric He other noble gases should be introduced into the CO₂-rich well-gas sample. A simple mass balance shows that air contamination would add more ³⁶Ar than the actual concentration in the gas sample⁶. Suppose that the He is, in fact, a mixture of MORB-type He and radiogenic He formed in the continental crust; accordingly a simple mass balance

$$(\alpha)_{\text{CO}_2} = a(\alpha)_{\text{MORB}} + (1-a)(\alpha)_{\text{cont. crust}}$$

where α is either (⁴He/³He) or (³He/⁴He), shows us that the

Table 1 Isotope noble-gas signatures

	$\frac{4}{3}$	$\frac{20}{22}$	$\frac{21}{22}$	$\frac{40}{36}$	$\frac{129}{130}$	$\frac{136}{130}$	Ref.
Continental crust	2.5×10^7	9-0.2	0.03-0.7	2,000-140,000	6.48	2.3-7.9	10, 20
CO ₂ -rich well gas	228,000	11.6	0.062	3,000-34,000	6.65-7.18	2.23-2.46	3-6, 12
MORB	82,000	10-13	0.03-0.07	13,000-28,000	6.6-7.5	2.25-2.55	9-11, 13, 16, 20
Atmosphere	722,500	9.8	0.029	296	6.48	2.17	21

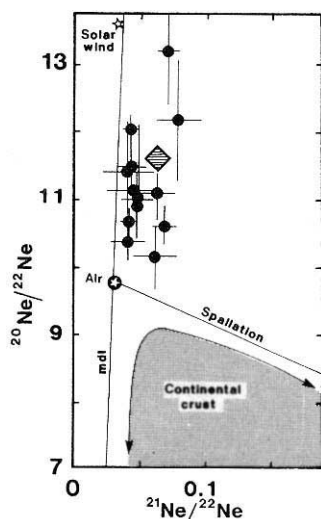


Fig. 1 Ne three-isotope plot. Included are data for MORB glasses ($\bullet$)²⁰ and CO₂-rich well gas ($\diamond$)⁶. Also indicated are solar Ne, the mass-discrimination line for Ne (mdl), the mixing line between spallogenic Ne and atmosphere, and the field for Ne from acidic rocks²⁰ whose characteristics show nuclear effects of the ²³⁸U-decay.

MORB-type component represents 99.4% of ³He and 36% of ⁴He, and the continental-crust component accounts for 0.6% of ³He and 64% of ⁴He.

Neon isotope systematics in CO₂-rich well gases have never been satisfactorily explained⁶. ²⁰Ne/²²Ne and ²¹Ne/²²Ne ratios range from 9.96 (ref. 5) to 11.6 (ref. 6) and 0.0302 (ref. 5) to 0.062 (ref. 6), respectively, and are both enhanced relative to air.

Although enhanced ²¹Ne/²²Ne ratios are well-known results of the nuclear reactions, for example, ¹⁸O (α, n) ²¹Ne and ²⁴Mg (n, α) ²¹Ne, caused by U-decay⁷, the same phenomenon causes low ²⁰Ne/²²Ne ratios (Table 1), thus defining the 'continental-crust' field in an Ne three-isotope plot (Fig. 1).

In MORB glasses, different systematics are observed. Figure 1 shows that all ²⁰Ne/²²Ne ratios for typical MORB glasses are above the atmospheric point. The same phenomenon has been observed for neon in diamonds⁸. The cause of this characteristic is not yet well known. Possibly, either, (1) neon from the lower mantle—with a different isotopic composition, close to solar Ne—is admixed to the MORB reservoir under the ridges, thus creating a mixing line comparable to noble gas isotope correlations reported by Allègre *et al.*^{9,10} or (2) the early atmosphere lost some of its neon: through mass fractionation effects, the atmospheric point shifted down along the mass discrimination line to its present value so the variation in ²⁰Ne/²²Ne in MORB glasses (Fig. 1) then only represents a random distribution around a 'mantle pole'.

Through geological time, nuclear reactions due to ²³⁸U-decay then enriched the MORB source in ²¹Ne, thus shifting it to the

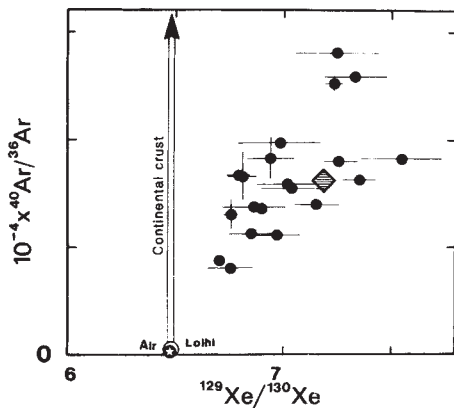


Fig. 2 Shown are the ⁴⁰Ar/³⁶Ar versus ¹²⁹Xe/¹³⁰Xe ratios for MORB and CO₂-rich well gas. The vertical line represents the evolution line for material derived from the continental crust. Loihi represents lower mantle material, measured by crushing under ultra-high vacuum and by stepwise heating of basalt glasses¹⁴. The Harding County data point is from Phinney *et al.*⁶.

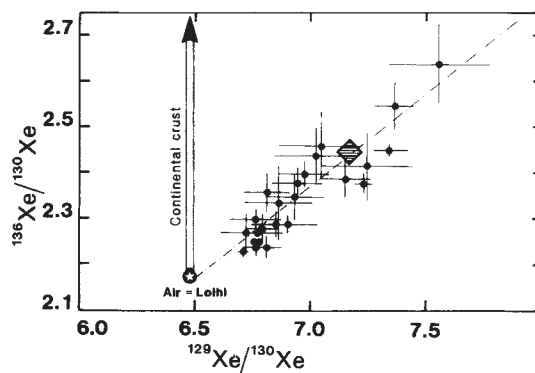


Fig. 3 ¹³⁶Xe/¹³⁰Xe versus ¹²⁹Xe/¹³⁰Xe in MORB and CO₂-rich well gas. Both the correlation between radiogenic isotopes and the excellent agreement between data for MORB and the CO₂-rich well gas are remarkable.

right-hand side of the mass discrimination line. Whatever the explanation is, the CO₂-rich well gas Ne (ref. 6) is plotted perfectly in the MORB area, making any neon contribution from continental crust unlikely.

Zartman *et al.*¹ published ⁴⁰Ar/³⁶Ar ratios in CO₂-rich well gases from Harding County of 22,500 and 34,000 (Mitchell no. 4 and no. 12) whereas Phinney *et al.*⁶ reported a value of 16,200. Such high values are typically observed in MORB glasses⁹⁻¹¹ and in felsic rocks¹⁰. Thus from Ar data alone, contamination by continental crust cannot be excluded, but if we combine two radiogenic isotopes, such as ⁴⁰Ar and ¹²⁹Xe (Fig. 2), then the point for the CO₂-rich well gas is within the data field of MORB samples. Any admixing of radiogenic ⁴⁰Ar from the continental crust should shift the point parallel to the continental crust evolution line upwards to higher ⁴⁰Ar/³⁶Ar ratios. This, however, is not so and Fig. 2 provides a good argument against a significant noble gas contribution from continental crust.

The best Xe data for CO₂-rich well gases are given by Hennecke and Manuel⁵, Phinney *et al.*⁶ and Smith and Reynolds¹². I have chosen the data with the highest ¹²⁹Xe excess⁶ for this comparison. Figure 3 shows ¹³⁶Xe/¹³⁰Xe versus ¹²⁹Xe/¹³⁰Xe for typical MORB glasses (refs 13, 9, 14 and Staudacher *et al.*, in preparation). Again the point for CO₂-rich well gas falls perfectly within the area defined by MORB samples. Any contribution of Xe derived from the spontaneous fission of ²³⁸U from the continental crust would shift the point vertically upwards, parallel to the continental-crust evolution line. This is not so and the excellent agreement of MORB data and data for CO₂-rich well gas show that a continental-crust component in the latter is absent.

Zartman *et al.*¹ published $\delta^{13}\text{C}$ values for a large number of well gases. The CO₂-rich well gases from Harding County are quite different from most other gases, showing $\delta^{13}\text{C}$ of -3.9 and -4.1‰ (Mitchell nos 4 and 12) relative to the PDB standard. Such $\delta^{13}\text{C}$ values are typical for CO₂ within vesicles of MORB glasses¹⁵ and confirm the similarity of the isotope signatures of CO₂-rich well gases and gases in MORB samples.

In conclusion, it can be said that CO₂-rich well gases and gases from MORB glasses show, except for He, essentially identical isotope signatures. The source region of both is probably the same. CO₂-rich well gases seemingly contain little or no gases derived from the continental crust, apart from some He. Such a He component is not surprising because of the high diffusion coefficient of He relative to other noble gases and its high concentration in continental-crustal material. For Ne, Ar, Xe and CO₂ such a continental-crustal component can be largely excluded.

Also, Phinney *et al.*⁶ concluded, based on the isotopic composition of Xe from CO₂-rich well gases, that the spontaneous fission of ²⁴⁴Pu is responsible for less than 20% of the fissiogenic

^{136}Xe excess. Because of the mantle origin of Harding County CO_2 -rich well gases, this conclusion can be directly adopted for MORB noble gases.

This conclusion is important because with the published Xe data^{9,10,13,16} on MORB glasses, it had not been possible to show without ambiguity if, and to what degree, the spontaneous fission of ^{244}Pu accounted for the fissiogenic Xe excesses in typical MORB glasses.

The noble-gas isotope signature for CO_2 -rich well gases described above is difficult to reconcile with the conclusion by Ozima *et al.*¹⁷ that "the source region for the ^{129}Xe excess must have been isolated for almost the whole history of the Earth from the atmosphere and/or the rest of the mantle from which the atmosphere was generated". This conclusion is based on a "xenon isotope evolution model" for the Earth as illustrated in ref. 17, Fig. 4, which uses for a model the evolution of $^{136}\text{Xe}/^{130}\text{Xe}$ and $^{129}\text{Xe}/^{130}\text{Xe}$ due to spontaneous fission of ^{244}Pu and decay of ^{129}I with the time of atmosphere outgassing (t_d) as a variable parameter. The evolution curves in their model are constrained by two points: A is the Xe isotopic composition of the present atmosphere, A_0 is the isotopic composition of the initial, non-radiogenic, non-fissiogenic atmosphere (U-Xe), estimated by Pepin and Phinney¹⁸, which itself is largely model-dependent. In ref. 17 (Fig. 4) the data point for CO_2 -rich well gas corrected for ^{136}Xe due to ^{238}U spontaneous fission falls in a forbidden area, below the dotted evolution curve ($t_d \rightarrow 0$), thus leading Ozima *et al.* to the above-mentioned conclusion.

If the source of CO_2 -rich well gases or MORB was in fact isolated from the mantle from which the atmosphere was formed, then the atmosphere could not have been generated from the upper mantle, the *de facto* source of MORB. Such a hypothesis completely contradicts all known models of atmosphere formation.

If CO_2 -rich well gases come from the deep mantle as Ozima *et al.* suggest, then the ^{129}Xe excesses, fission Xe excesses, high $^{40}\text{Ar}/^{36}\text{Ar}$ ratios, Ne anomalies and even radiogenic He should also come from the deep mantle. This contradicts the observations that the deep or lower mantle is less radiogenic^{19,9,10,14}.

A more plausible solution to this puzzle would be that the proportion of fissiogenic ^{136}Xe in the atmosphere that is due to spontaneous fission of ^{244}Pu ¹⁸ is highly overestimated and that the $^{244}\text{Pu}/^{129}\text{I}$ ratio given by Ozima *et al.* should be smaller. The U-Xe-point A_0 in ref. 17, Fig. 4 would lie higher, above $^{136}\text{Xe}/^{130}\text{Xe} = 2.1$, the evolution curves would be more flat and less curved. The ' ^{238}U -corrected' point for CO_2 -rich well gas would agree with the 'xenon isotope evolution model', implying that the atmosphere, CO_2 -rich well gases and the highly radiogenic noble gases in MORB are all generated from the same source, the upper mantle.

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- Zartman, R. E., Wasserburg, G. J. & Reynolds, J. H. *J. geophys. Res.* **66**, 277-306 (1961).
- Butler, W. A., Jeffrey, P. M. & Reynolds, J. H. *J. geophys. Res.* **68**, 3283-3291 (1963).
- Wasserburg, G. J. & Mazor, E. *Am. Assoc. Pet. Geol., Mem* **4**, 386 (1965).
- Boulos, M. S. & Manuel, O. K. *Earth planet. Sci. Lett.* **174**, 1334-1336 (1971).
- Hennecke, E. W. & Manuel, O. K. *Earth planet. Sci. Lett.* **27**, 346-355 (1975).
- Phinney, D., Tennyson, J. & Frick, U. *J. geophys. Res.* **83**, 2313-2319 (1978).
- Wetherill, G. W. *Phys. Rev.* **96**, 679-683 (1954).
- Honda, M. & Reynolds, J. H. *Abstract Eos*, **66** (46), 1117 (1985).
- Allègre, C. J., Staudacher, Th., Sarda Ph. & Kurz, M. *Nature* **303**, 762-766 (1983).
- Allègre, C. J., Staudacher, Th. & Sarda, Ph. *Earth planet. Sci. Lett.* **81**, 127-150 (1987).
- Sarda Ph., Staudacher, Th. & Allègre, C. J. *Earth planet. Sci. Lett.* **72**, 357-375 (1985).
- Smith, S. P. & Reynolds, J. H. *Earth planet. Sci. Lett.* **54**, 236-238 (1981).
- Staudacher, Th. & Allègre, C. J. *Earth planet. Sci. Lett.* **60**, 389-406 (1982).
- Staudacher, Th., Kurz, M. D. & Allègre, C. J. *Chem. Geol.* **56**, 193 (1986).
- Pineau, F. & Javoy, M. *Earth planet. Sci. Lett.* **62**, 239-257 (1983).
- Barraclough, B. L. & Marti, K., *Abstract Eos* **66** (46), 1117 (1985).
- Ozima, M., Podosek, F. A. & Igarashi, G. *Nature* **315**, 471-474 (1985).
- Pepin, R. O. & Phinney, D. *Moon Planets* (in press).
- Hart, R. & Hogan, L. in *Terrestrial Rare Gases* Vol. 193 (eds Alexander, E. C. & Ozima, M.) (Japan Scientific Society Press, Tokyo, 1978).
- Sarda Ph., Richardson S., Staudacher, Th. & Allègre, C. J. *Abstract Eos*, **67**, 411 (1986).
- Nier, A. O. *Phys. Rev.* **79**, 450 (1950).

Short-lived radioactive disequilibria and magma dynamics in Etna volcano

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The interest of radioactive disequilibria for the study of magmatic processes has long been recognised¹. The use of ^{230}Th - ^{238}U disequilibria has been growing in recent years²⁻⁴; but, since the pioneering work by Capaldi *et al.*⁵, very few studies have been concerned with a systematic use of short-lived isotopes of the radioactive series (for example, ^{226}Ra , ^{210}Pb , ^{210}Po in the ^{238}U series, and ^{228}Ra , ^{228}Th in the ^{232}Th series). Here we present new precise results on the short-lived disequilibria in some recent lavas from Etna volcano. Our data show that a Ra enrichment took place in the Etna magma between 100 and 8,000 yr ago. Moreover, there is a clear correlation between ($^{226}\text{Ra}/^{230}\text{Th}$) activity ratios and the degree of differentiation of the magma, as indicated by its Th content. This correlation is interpreted as the result of mixing of a differentiated magma and a deep basic magma with a high ($^{226}\text{Ra}/^{230}\text{Th}$) ratio. The Ra and U enrichments relative to Th in recent volcanics could be a consequence of fluid-transfers in the mantle sources or in crustal magma reservoirs.

^{226}Ra - ^{230}Th and ^{228}Ra - ^{232}Th disequilibria are of a particular interest, because both concern the same pair of chemical elements with very different half-lives for ^{226}Ra (1,600 yr) and ^{228}Ra (5.77 yr): so radioactive equilibrium will be reached at a different rate for each pair. If, for example, ($^{226}\text{Ra}/^{230}\text{Th}$) = 1 and ($^{228}\text{Ra}/^{232}\text{Th}$) = 1 in a volcanic rock (both pairs in radioactive equilibrium), this means either no fractionation at all between Ra and Th or a fractionation more than 8,000 years old. (Throughout this paper, ratios in parentheses denote activity ratios.) If ($^{226}\text{Ra}/^{230}\text{Th}$) $\neq$ 1 and ($^{228}\text{Ra}/^{232}\text{Th}$) = 1, the Ra-Th fractionation must have occurred between 30 yr and 8,000 yr ago. If ($^{226}\text{Ra}/^{230}\text{Th}$) $\neq$ 1 and ($^{228}\text{Ra}/^{232}\text{Th}$) $\neq$ 1, the fractionation is less than 30 yr old. In this latter case, it is possible to date the fractionation event precisely, provided that the initial equilibrium conditions in the magma are known. The results can be discussed in a ($^{228}\text{Ra}/^{226}\text{Ra}$) against ($^{232}\text{Th}/^{226}\text{Ra}$) diagram, which is similar to the well-known ($^{230}\text{Th}/^{232}\text{Th}$) against ($^{238}\text{U}/^{232}\text{Th}$) diagram proposed by Allegre⁶.

The analytical results on some recent lava flows from Mount Etna are reported in Table 1. Major element compositions of the analysed rocks are given in Table 2. All the samples are trachybasalts or basaltic hawaiites, locally known as etnaïtes⁷. U and Th contents have been measured by isotope dilution and mass spectrometry, ($^{230}\text{Th}/^{232}\text{Th}$) ratios by α -spectrometry. ^{226}Ra , ^{228}Ra , ^{228}Th , ^{210}Pb have been measured by non destructive γ -spectrometry on a powdered sample of about 200 g, using a high-sensitivity (≈ 0.1 p.p.m. U) Ge HP(P) γ -ray spectrometer at the ISN (Nuclear Sciences Institut) in Grenoble⁸. More details about the method and the spectrometry system will be presented elsewhere.

The results of Table 1 show that only ($^{226}\text{Ra}/^{230}\text{Th}$) ratios are significantly different from unity. ^{226}Ra results are in good agreement with previous data on Etna lava flows^{5,9,10}. ^{228}Ra and ^{228}Th are in equilibrium with ^{232}Th in our samples, in contrast with the excesses reported by Capaldi *et al.*⁵.

Table 1 U and Th contents and activity ratios of the various nuclides of the ^{238}U and ^{232}Th series

Samples	U p.p.m.	^{238}U d.p.m./g	Th p.p.m.	^{232}Th d.p.m./g	$\left(\frac{^{238}\text{U}}{^{232}\text{Th}}\right)$	$\left(\frac{^{230}\text{Th}}{^{232}\text{Th}}\right)$	$\left(\frac{^{230}\text{Th}}{^{238}\text{U}}\right)$	$\left(\frac{^{226}\text{Ra}}{^{230}\text{Th}}\right)$	$\left(\frac{^{210}\text{Pb}}{^{226}\text{Ra}}\right)$	$\left(\frac{^{228}\text{Ra}}{^{232}\text{Th}}\right)$	$\left(\frac{^{228}\text{Th}}{^{232}\text{Th}}\right)$
1950	2.79	2.07	9.97	2.43	0.849	0.934	1.10	1.73	1.18	0.99	1.01
1964	2.50	1.85	8.96	2.19	0.847	0.943	1.12	1.88	1.00	1.02	0.99
1974	2.11	1.56	7.11	1.73	0.900	0.969	1.08	2.56	1.00	1.02	1.01
1978	2.30	1.70	7.75	1.89	0.900	0.96*	1.06	2.35	1.22	nd	nd
1979	2.52	1.87	8.81	2.15	0.868	0.947*	1.09	2.02	1.11	0.99	1.01
1983	2.41	1.79	8.29	2.02	0.882	0.935	1.06	2.23	1.20	0.98	1.04

The relative errors are estimated at 0.5% for U and Th contents (mass spectrometry), 1% for $(^{238}\text{U}/^{232}\text{Th})$ ratios, 1% or less for $(^{230}\text{Th}/^{232}\text{Th})$ ratios (1σ α counting statistics), 2% for $(^{230}\text{Th}/^{238}\text{U})$, 4–5% for $(^{226}\text{Ra}/^{230}\text{Th})$, $(^{228}\text{Ra}/^{232}\text{Th})$ and $(^{228}\text{Th}/^{232}\text{Th})$ ratios, 10–20% for $(^{210}\text{Pb}/^{226}\text{Ra})$ ratios. *, Results from ref. 4. See Table 2 for a brief description of the samples.

The existence of a ^{226}Ra enrichment in volcanic rocks from Etna was first reported by Cheminée¹¹ in 1973. The lack of any disequilibrium between ^{228}Ra and ^{232}Th shows that the Ra–Th fractionation took place between about 30 yr and 8,000 yr ago. Moreover, as a Pb–Ra fractionation probably occurred at the time of Ra–Th fractionation, the ^{210}Pb – ^{226}Ra equilibrium indicates an event older than 100 years. In other words the time of transfer of the magma between its Ra enrichment and eruption is in the range 100–8,000 years. If the Ra enrichment was a continuous process at a rate slow enough so as not to affect the ^{228}Ra – ^{232}Th and ^{210}Pb – ^{226}Ra equilibria, then it should have begun much more than 100 years ago.

The large Ra–Th fractionation ($(^{226}\text{Ra}/^{230}\text{Th}) \approx 2$) is unlikely to be due to solid–liquid partition processes during partial melting or fractional crystallization. Such a Ra enrichment in the magma would only be possible if Ra was much more incompatible than Th ($D_{\text{Ra}} \ll D_{\text{Th}}$). In solid–liquid partition processes, the behaviour of Ra is likely to be similar to that of Ba, which does not seem more incompatible than Th in Etna magmas⁴. But Ra could be a relatively mobile element, especially in the presence of fluids¹¹. Ra is indeed strongly enriched in CO_2 rich carbonatite magmas¹².

The Ra enrichment in Etna may thus be the result of a contamination of the magma by fluid transfer. Such a mechanism was proposed to explain enrichments in K, Rb, Cs in some very recent lavas from Etna, mainly since the 1974 eruption¹³. Indeed the 1974, 1978, 1983 and, to a lesser extent, 1979 lavas are surprisingly rich in K, Rb, Cs compared to the more differentiated 1950 and 1964 lavas (ref. 13, and Table 2). $^{87}\text{Sr}/^{86}\text{Sr}$ ratios

are also slightly different: 0.70345 ± 2 for 1950 and 1964 lava flows: 0.70358 ± 2 for 1978 and 1974 flows (unpublished results). These anomalies have been interpreted as the result of a selective contamination of the magma at depth by the sedimentary basement of Etna¹³. The samples of the 1974 and 1978 lava flows have also the highest $(^{226}\text{Ra}/^{230}\text{Th})$ ratios (2.56 and 2.35 respectively, see Table 1). However, the 1950 and 1964 samples still have a strong ^{226}Ra – ^{230}Th disequilibrium, although they appear little affected by a contamination in alkaline elements. If this latter contamination and the Ra enrichment are the result of a common process ^{226}Ra appears to be the most sensitive tracer. Moreover, as already stated, this contamination should have occurred in the magma more than 100 yr ago and not during the intervals between successive eruptions.

It is worth noting that the variations in ^{226}Ra contents and $(^{226}\text{Ra}/^{230}\text{Th})$ ratios are clearly related to the degree of differentiation of the lava samples, as indicated by the good correlation between their ^{226}Ra and Th contents (Fig. 1 inset). The Ra enrichment is greater in the less differentiated lavas, as the 1974 sample, and the ^{226}Ra activities slightly decrease in the more differentiated lavas. This 1974 Ra-rich magma is likely to be close in composition to the deep Etna magma, which has already experienced some differentiation in a deep reservoir⁴. The inverse correlation between the Ra enrichment and the Th content could then result from mixing of two different magmas: a deep magma with low Th content and high $(^{226}\text{Ra}/^{230}\text{Th})$ ratio, and a shallow, more differentiated magma with a higher Th content and low $(^{226}\text{Ra}/^{230}\text{Th})$ ratio. This mixing model is strongly supported by the very good linear correlation in a $(^{226}\text{Ra}/^{230}\text{Th})$ against $1/(^{230}\text{Th})$ diagram (Fig. 1). The basic magma and the more evolved one display also small but significant differences in their $(^{230}\text{Th}/^{232}\text{Th})$ and $(^{238}\text{U}/^{232}\text{Th})$ ratios (Table 1). The differentiated magma would have stayed in the upper part of the volcanic 'plumbing system' for a period long enough to differentiate and would have been mixed in recent years with a new basic magma rising from the deeper part of the magmatic system. This could explain the unusually intense volcanic activity of Mount Etna since 1971⁷, and ^{226}Ra would be in this respect a very good tracer of basic magma reinjection. The $(^{226}\text{Ra}/^{230}\text{Th})$ value of a particular batch of magma erupting at the surface would depend on the relative proportions of the basic and differentiated magmas, and thus on the location and depth of its source in the volcanic 'plumbing system'. For example, the 1974 excentric eruption, which occurred at only 1,670 m above sea level, probably tapped a deep part of the volcanic feeding system and the 1974 lavas are thus the most representative of the deep magma. The subsequent eruptions tapped a 'mixed' magma from a shallower part of the volcanic system^{7,14}. This explains the irregularities in the $(^{226}\text{Ra}/^{230}\text{Th})$ ratios variation through time.

The lower $(^{226}\text{Ra}/^{230}\text{Th})$ ratio of the differentiated magma can be explained in two ways: either this magma derives from a basic magma less ^{226}Ra enriched than the present one, or its $(^{226}\text{Ra}/^{230}\text{Th})$ ratio has decreased by radioactive decay during its stay at depth. A simple closed-system calculation, assuming

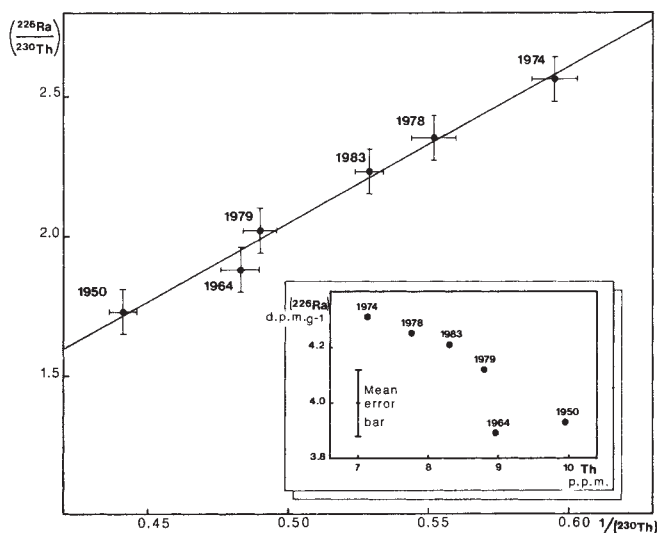


Fig. 1 Variation of $(^{226}\text{Ra}/^{230}\text{Th})$ ratios against $1/(^{230}\text{Th})$ in recent Etna lavas. The linear correlation is interpreted by a mixing model (see text). Inset: Variation of the (^{226}Ra) activities as a function of Th content of the same samples.

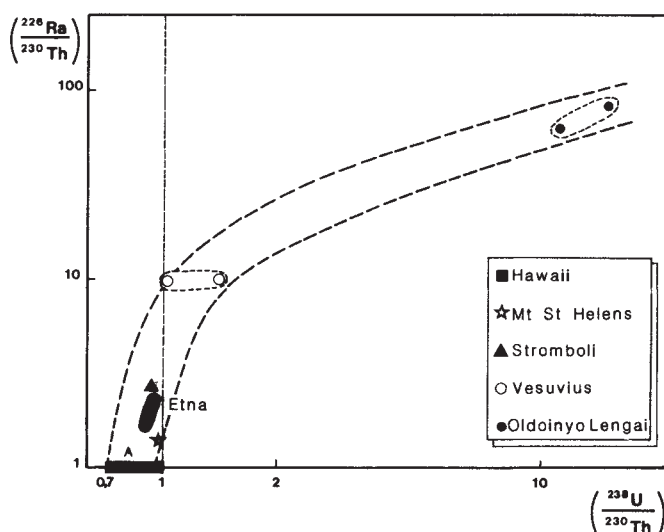


Fig. 2 Possible relationship between $(^{226}\text{Ra}/^{230}\text{Th})$ and $(^{238}\text{U}/^{230}\text{Th})$ ratios in recent lavas from different active volcanoes. High $(^{226}\text{Ra}/^{230}\text{Th})$ and $(^{238}\text{U}/^{230}\text{Th})$ ratios indicate an increasing role of fluids. The domain A shows the range in $(^{238}\text{U}/^{230}\text{Th})$ ratios of most young volcanic rocks (see text). Data from refs 12, 15, 17–19.

no Ra–Th fractionation during differentiation, shows that it would take about 1,740 years to lower this ratio from 2.56 (1974) to 1.73 (1950). This is of course a maximum value since a Ra–Th fractionation produces a decrease in Ra/Th ratios.

The question remains about the origin of the Ra enrichment in the deep Etna magma. If a permanent reservoir has been present under Etna for more than 10^5 years⁴, ^{226}Ra and ^{230}Th should be in equilibrium in this reservoir. Then the Ra enrichment must have occurred in this reservoir, or at shallower levels, by a selective contamination induced by fluids percolating through the wall-rocks. It is also possible that a new magma was recently injected in the deep reservoir: in that case the Ra enrichment may have its source at greater depths or even in the zone of partial melting, as proposed for Vesuvius by Capaldi *et al.*¹⁵. If the fluids play an essential part in the Ra enrichment process, other mobile elements, such as K, Rb, Cs and, to a lesser extent, U, are likely to be enriched in the same way.

When considering the few available results on recent volcanics around the world, it is clear that the most Ra enriched products are also the most U enriched¹². A measure of this relative enrichment compared to Th is given by the $(^{238}\text{U}/^{230}\text{Th})$ ratio. Allegre and Condomines¹⁶ have shown that most recent volcanic rocks have $(^{238}\text{U}/^{230}\text{Th})$ ratios lower than 1 or very close to 1; there are only a few examples of volcanic rocks having $(^{238}\text{U}/^{230}\text{Th})$ ratios higher than unity: they belong either to continental high K-alkaline volcanics or to calco-alkaline vol-

Table 2 Major elements analyses of the Etna samples (analyses by M. Lenoble, Laboratoire de Pétrographie, Paris 6 University)

	1950	1964	1974	1978	1979	1983
SiO ₂	47.98	48.17	47.16	47.87	47.92	47.41
Al ₂ O ₃	17.50	16.13	16.56	16.40	17.05	17.08
Fe ₂ O ₃	5.24	3.75	4.58	4.77	3.30	3.19
FeO	5.28	7.12	6.35	6.43	7.13	7.36
MgO	4.96	5.93	5.82	5.76	5.36	5.87
CaO	10.17	10.45	11.05	10.59	10.46	10.52
Na ₂ O	4.31	3.94	3.56	3.66	3.97	3.81
K ₂ O	1.73	1.69	1.99	2.04	1.87	1.93
MnO	0.19	0.17	0.17	0.18	0.17	0.18
TiO ₂	1.65	1.71	1.68	1.68	1.64	1.72
P ₂ O ₅	0.54	0.52	0.52	0.58	0.52	0.54
H ₂ O ⁺	0.07	0.36	0.65	0.36	0.43	0.14
H ₂ O ⁻	0.13	0.03		0.03	0.10	0.06
Total	99.75	99.97	100.09	100.35	99.92	99.81

In the following description, the numbers in parentheses are those of the samples in refs 7, 14.

1950 (50–605), front of the mid-December 1950 flow, east 800 m above sea level (a.s.l.). 1964 (64–09), May 1964 south lava flow, south 3,000 m (a.s.l.). 1974 (74–1379), 25 March 1974 scoria from Mt de Fiore II, west 1,670 m (a.s.l.). 1978 (78–07), April 1978 lava flow, upper part of the south-east fissure, south-east 3,000 m (a.s.l.). 1979 (79–02), front of the 6 August 1979 lava flow, east-north-east 1,780 m (a.s.l.). 1983 (83–26), 1983 lava flow, sampled on 20 June, south 2,300 m (a.s.l.).

canics; in this latter case, their U enrichment is thought to be the result of metasomatism of the mantle source through U rich fluids¹⁶. It is most significant that the lavas showing the largest ^{226}Ra – ^{230}Th disequilibrium also have $(^{238}\text{U}/^{230}\text{Th})$ ratios higher than 1: this is the case for the carbonatites studied by Williams *et al.*¹² [$(^{226}\text{Ra}/^{230}\text{Th}) = 83$, $(^{238}\text{U}/^{230}\text{Th}) = 15$] and for the Vesuvius lavas¹⁵. Using the available results, one may infer a rough correlation between $(^{226}\text{Ra}/^{230}\text{Th})$ and $(^{238}\text{U}/^{230}\text{Th})$ ratios (Fig. 2), which would be indicative of an increasing role of fluids towards high $(^{226}\text{Ra}/^{230}\text{Th})$ and $(^{238}\text{U}/^{230}\text{Th})$ ratios. In other words, in a 'dry' environment the magma would show $(^{238}\text{U}/^{230}\text{Th}) < 1$ and $(^{226}\text{Ra}/^{230}\text{Th}) \approx 1$, and in a 'wet' one $(^{238}\text{U}/^{230}\text{Th}) > 1$ and $(^{226}\text{Ra}/^{230}\text{Th}) \gg 1$. A moderate role of fluids, as in Etna, would be indicated by $(^{238}\text{U}/^{230}\text{Th}) \leq 1$ and $(^{226}\text{Ra}/^{230}\text{Th}) > 1$. Of course the composition of the fluids is likely to have an influence on the degree of Ra and U enrichment and CO₂-rich fluids, as in carbonatites, may have a predominant role.

Clearly, it is not possible at present to proceed much further in the discussion about the ultimate origin of the Ra enrichment in the Etna magma. However, we can study the kinetics of the process (continuous or episodic) by analysing old historic lava flows of well-known age. The evolution of the $(^{226}\text{Ra}/^{230}\text{Th})_0$ initial ratios through time should indeed give fundamental information about magma dynamics for the past few centuries.

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- Oversby, V. & Gast, P. W. *Earth planet. Sci. Lett.* **5**, 199–206 (1968).
- Allegre, C. J. & Condomines, M. *Earth planet. Sci. Lett.* **28**, 395–406 (1976).
- Condomines, M. & Allegre, C. J. *Nature* **288**, 354–357 (1980).
- Condomines, M., Tanguy, J. C., Kieffer, G. & Allegre, C. J. *Geochim. cosmochim. Acta* **46**, 1397–1416 (1982).
- Capaldi, G., Cortini, M., Gasparini, P. & Pece, R. *J. geophys. Res.* **81**, 350–358 (1976).
- Allegre, C. J. *Earth planet. Sci. Lett.* **5**, 209–210 (1968).
- Tanguy, J. C. thesis Univ. Pierre et Marie Curie (1980).
- Ma, J. L. thesis Grenoble (1984).
- Le Cloarec, M. F., Lambert, G., Le Guern, F. & Ardouin, B. *C.r. hebdo. Séanc. Acad. Sci., Paris* **298**, II, 305–308 (1984).

- Lambert, G., Le Cloarec, M. F., Ardouin, B. & Le Rouley, J. C. *Earth planet. Sci. Lett.* **76**, 185–192 (1985–86).
- Cheminee, J. L. thesis Univ. Grenoble (1973).
- Williams, R. W., Gill, J. B. & Bruland, K. W. *Geochim. cosmochim. Acta* **50**, 1249–1259 (1986).
- Clocchiatti, R., Joron, J. L. & Treuil, M. *IAVCEI Scientific Assembly (Abstr.)* (1985).
- Tanguy, J. C. & Clocchiatti, R. *Bull. Volc.* **47**, 4 (1984).
- Capaldi, G., Cortini, M. & Pece, R. *J. Volc. geotherm. Res.* **14**, 247–260 (1982).
- Allegre, C. J. & Condomines, M. *Nature* **299**, 21–24 (1982).
- Bennett, J. T., Krishnaswami, S., Turekian, K. K., Melson, W. G. & Hopson, G. A. *Earth planet. Sci. Lett.* **60**, 61–69 (1982).
- Krishnaswami, S., Turekian, K. K. & Bennett, J. T. *Geochim. cosmochim. Acta* **48**, 505–511 (1984).
- Capaldi, G., Cortini, M. & Pece, R. *Isotope Geoscience* **1**, 39–55 (1983).

Structure of the Bushveld Complex from resistivity measurements

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Knowledge of the deep structure of the arc-like bodies constituting the Bushveld Complex (South Africa) is essential not only to understand the emplacement history of this huge layered intrusion, but also for success in the exploration for the platiniferous Merensky Reef and chromitite layers occurring near its base. Despite many studies the structure of the complex has remained obscure. Here we describe new insights into the structure and additional geophysical boundary conditions, based on a geoelectrical study. This has provided a geoelectrical stratigraphy according to which a conductive layer below the granitic rocks, of both the eastern and western compartments of the Complex, is correlated with sedimentary rocks of the Transvaal Sequence over large areas, rather than with the mafic sequence as conventionally assumed. A new feature of this model is that the mafic sequence in both compartments occurs as segments of an inward-dipping sheet transgressing the layering of the sedimentary rocks into which it intruded. The geoelectrical model supports earlier gravity models in that mafic rocks are absent from the central part of the Complex, but shows that here the Bushveld granite directly overlies Archaean basement rather than Transvaal Sequence rocks as previously accepted.

The structure of the Bushveld Complex has been under scrutiny since its discovery. Cousins¹ showed that the generally accepted lopolith model² could not be reconciled with gravity data, and proposed an alternative model in which the Complex was thought to consist of discrete compartments. In this model the Lebowa Granite Suite (LGS)³ in the central part of the Complex is underlain by sedimentary strata of the Transvaal Sequence and not by the heavy mafic rocks of the Rustenburg Layered Sequence (RLS)³. Modelling by Smit^{4,5} of gravity data on the central part of the Complex follows the model proposed by Cousins¹. Several models for the western and eastern compartments of the Complex published subsequently⁶⁻¹⁰, based mainly on gravity data, are unanimous that the mafic RLS directly underlies the LGS, although it does not outcrop towards the centre of the ring-like structure.

The results of ~150 deep direct current resistivity soundings on the geoelectrically ideally stratified Bushveld Complex and

Table 1 Geoelectrical stratigraphy

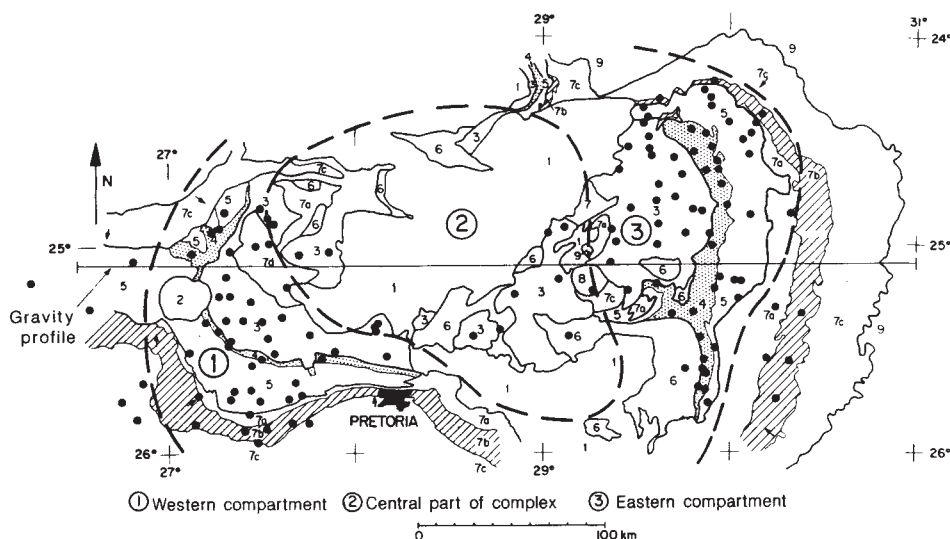
Geology	Resistivities in model calculations (Ω m)	Thickness in model calculations (m)
Lebowa Granite Suite (LGS)	22,000	2,250
Rustenburg Layered Suite (RLS)	400	20,000*
{ upper zone	20,000	3,500
{ main zone		
{ critical zone	3,000	3,000
{ lower zone		
Transvaal Sequence	1,000-10,000	6,100
{ upper part of Pretoria Group	2,000	2,000
{ Silverton Formation		
{ graphitic member of Silverton Formation	1	>1,000

* Pseudo-thickness due to anisotropic behaviour of alternating layers of conductive magnetite and resistive gabbroic rocks. The true thickness is about 2,200 m¹⁴.

the older Transvaal Sequence (Fig. 1) have led to new constraints being placed on a structural model. In an otherwise electrically resistive environment, two conductive horizons—the one associated with the magnetite-bearing upper zone of the RLS and the other, a graphitic shale, situated in the Silverton Formation of the Pretoria Group, Transvaal Sequence—form ideal markers to study the structural relationships. Although both conductors have similar conductances (conductivity-thickness products), they can be distinguished by their average resistivities. Several soundings have shown that the average resistivity of the upper zone of the RLS, which contains ~24 very conductive but relatively thin magnetite layers interlayered with highly resistive gabbroic rocks, is ~400 Ω m, whereas that of the graphitic shale in the Silverton Formation is at least an order of magnitude less (Table 1, Fig. 2). Soundings on the LGS of the eastern and western compartments invariably indicate the presence of a good conductor at depth¹¹.

Theoretical sounding curves were calculated from average geoelectrical parameters for models, assuming the complete RLS as being either present below the LGS with decreasing dip towards the central part of the Complex (Fig. 2), or as a symmetrical intrusion with the subcrop of the RLS below the LGS (Fig. 3). These curves are displayed in Figs 2b and 3b. It is evident from these model curves that the presence of the complete RLS (Fig. 2, curve A), or even only parts of it (Fig. 3, curves B and C), below the LGS has the effect of shifting the

Fig. 1 Simplified geological map of the Bushveld Complex showing electrical resistivity sounding positions. Thick dashed lines indicate the boundaries of compartments and arrows indicate dip direction. The numbers represent the following geological units in order of increasing age: 1, post-Bushveld cover; 2, post-Bushveld Alkaline Complexes; 3, Lebowa Granite Suite (LGS); 4, upper zone (UZ) of Rustenburg Layered Suite (RLS); 5, main (MZ), critical (CZ) and lower zones (LZ) of RLS; 6, Rooiberg Group; 7, Transvaal—generalized (TVL); 7a, upper part of Transvaal Sequence (UTVL); 7b, Silverton Formation of Pretoria Group; 7c, lower part of Transvaal Sequence (LTVL); 7d, Crocodile River Fragment of Transvaal Sequence; 8, Elandsplaagte Dome of Groblersdal Group; 9, older basement rocks.



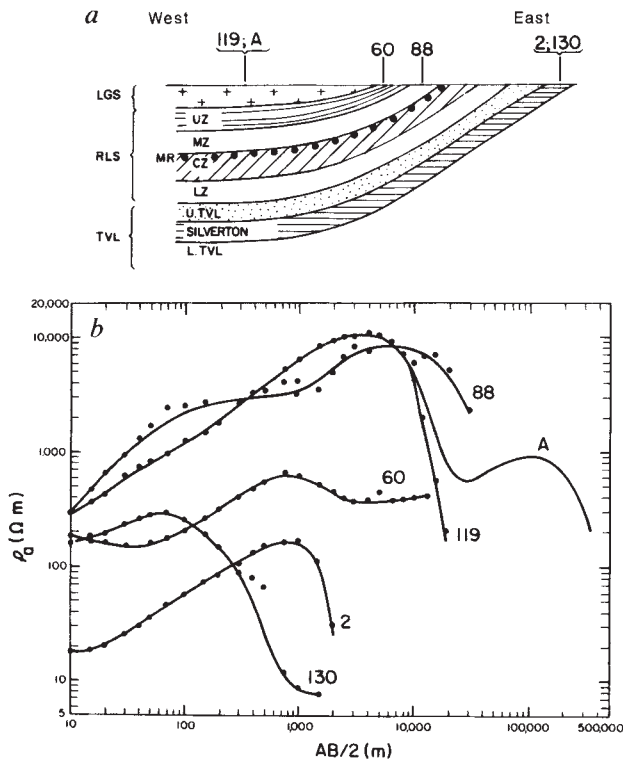


Fig. 2 A comparison of measured sounding curves (eastern compartment) with a calculated sounding curve (A) for a model in which the RLS has a gentle decreasing dip towards the centre of the Bushveld Complex. U.TVL and L.TVL refer to those formations of the Transvaal Sequence (TVL) above and below the Silverton Formation, respectively. The thickness of the LGS determined from sounding 119 was used in the calculation of curve A. *a*, Sounding positions for calculated curve (A) and field curves with assumed model; not to scale. MR, Merensky Reef (see legend to Fig. 1 for other symbols). *b*, Measured and calculated curves. Curve A was calculated according to average geoelectrical parameters established from 150 soundings.

descending branch of the sounding curve farther to the right than the measured curves and causing the descent of the curve to be not as steep as those actually measured. The most pronounced difference occurs when the upper zone of the RLS is present below the LGS (curve A). Geoelectrically, the thickness of the upper zone appears to be ≥ 10 times thicker than the true thickness, owing to the anisotropic behaviour of the alternating layers of very conductive magnetite ($\rho_{\text{average}} \approx 0.07 \Omega \text{ m}$) and resistive gabbro ($\rho_{\text{average}} \approx 10,000 \Omega \text{ m}$) comprising the upper zone. None of the sounding curves so far measured on the LGS conform to any of the calculated curves for the models presented in Figs 2 and 3, and these models are therefore not representative of the structure of the Bushveld Complex.

On the other hand, the presence of a very good conductor below the LGS of the eastern and western compartments is

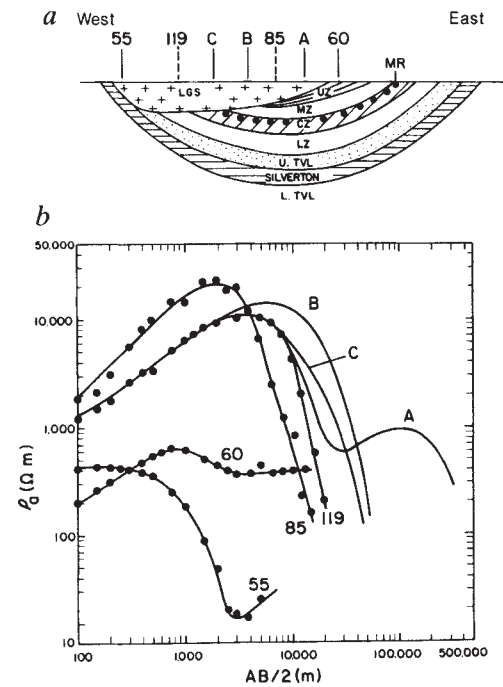
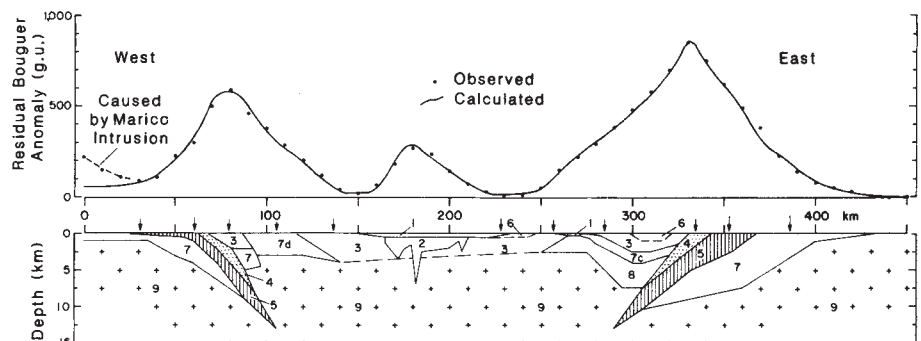


Fig. 3 A comparison of measured with calculated sounding curves for a model of the eastern compartment where the RLS is shown as a subcrop below the LGS. Feeder to the Complex not shown. *a*, Positions of measured (55, 60, 85, 119) and calculated (A, B, C) sounding curves; not to scale. Symbols as in Figs 1 and 2. *b*, Measured and calculated curves. Curves A, B and C were calculated according to average geoelectrical parameters and indicate the effect that the presence of different units of the RLS between the LGS and the Silverton Formation have on sounding curves. Sounding curves 85 and 119 are representative of the many curves measured on the LGS which all show the presence of a conductor below the resistive granite cover.

supported by the steep descent of the final segment of all sounding curves measured on these outcrops of the LGS. This, together with the fact that the final segments of several curves have resistivities $< 400 \Omega \text{ m}$, strongly suggests that the underlying conductor reflects the presence of the Silverton Formation. At sounding 55 (Fig. 3), for example, where it is known from nearby outcrops that the LGS is underlain by and in direct contact with the Silverton Formation, the latter has a resistivity of $< 15 \Omega \text{ m}$.

From the above arguments and the similarity in geoelectrical stratigraphy for both the western and eastern compartments, we postulate that the LGS, in the central parts of these compartments, is directly underlain by rocks of the Transvaal Sequence containing the highly conductive graphitic shale member. This, however, does not exclude the possible presence in these areas of the resistive Magaliesberg Quartzite Formation between the

Fig. 4 Observed and calculated Bouguer gravity anomaly for proposed structural model across an east-west section (Fig. 1) of the Bushveld Complex. Numbers in the lower half of the figure represent the same geological units as in Fig. 1. Arrows along profile indicate the projected positions of Schlumberger soundings.



LGS and the Silverton Formation, which, in the normal stratigraphic sequence, occurs above the Silverton Formation.

This interpretation is contrary to previous gravity models⁴⁻⁹ for these areas. The gravity data can, however, easily be reconciled with the geoelectrical model by treating the mafic sequence in the eastern and western compartments of the Complex as segments of an inward dipping circular sheet-like intrusion, transgressing the layering of the Transvaal Sequence at depth (Fig. 4). According to the better-constrained geoelectrical-gravity model the LGS attains a maximum thickness of 3.9 km and is directly underlain by a thick succession of Transvaal Sequence rocks in the western compartment. In the eastern compartment the LGS attains a maximum thickness of 2.6 km as determined from geoelectrical soundings¹¹, and is assumed to be underlain by the Transvaal Sequence resting on rocks of the older Groblersdal Group³. A notable disagreement between this first-order model and field geology is the reversed dips observed in the mafic rocks in a small area south-east of Groblersdal¹⁵.

Immediately to the east of the Crocodile River Fragment (Fig. 1) in the central part of the Complex, Transvaal Sequence rocks occur below the LGS, but further to the east the resistivity results show that a resistive layer ($\rho \approx 30,000 \Omega \text{ m}$), with a thickness of $\sim 2.4 \text{ km}$ occurs above a slightly less resistive zone ($\rho \approx$

$8,000 \Omega \text{ m}$) overlying a less resistive basement ($\rho \approx 3,000 \Omega \text{ m}$). The uppermost layer represents the LGS, whereas the second and third zones, because of their resistivity values associated with low gravity values, probably represent Archaean granitic basement. The final less resistive unit observed on the sounding curves corresponds to the medium resistivity zone present at a depth of 5–35 km below Archaean cratonic areas in southern Africa as described by Van Zijl¹². The sharp positive Bouguer anomaly in the central part is associated with a pre-Karoo, post-Bushveld Elandskraal-type volcanic centre¹³.

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1. Cousins, C. A. *Trans. geol. Soc. S. Afr.* **62**, 179–189 (1959).
2. Hall, A. L. *Geol. Surv. S. Afr., Mem.* **28**, 540pp. (1932).
3. South African Committee for Stratigraphy *Geol. Surv. S. Afr. Handb.* **8**, 690pp. (1980).
4. Smit, P. J. *Geol. Surv. S. Afr. open File rep.* **300**, 243pp. (1961).
5. Smit, P. J., Hales, A. L. & Gough, D. I. *Geol. Surv. S. Afr. Handb.* **3**, 486pp. (1962).
6. Molyneux, T. G. & Klinkert, P. S. *Trans. geol. Soc. S. Afr.* **81**, 359–368 (1978).
7. Walraven, F. & Darracott, B. W. *Trans. geol. Soc. S. Afr.* **79**, 22–26 (1976).
8. Hattingh, P. J. *Trans. geol. Soc. S. Afr.* **83**, 125–134 (1980).
9. Marlow, A. G. & Van der Merwe, M. J. *Trans. geol. Soc. S. Afr.* **80**, 117–124 (1977).
10. Sharpe, M. R. & Snyman, J. A. *Tectonophysics* **65**, 85–110 (1980).
11. De Beer, J. H., Meyer, R. & Hattingh, P. in *Proterozoic Lithospheric Evolution* (ed. Kröner, A.). (Geodynamics Series, Am. Geophys. Un., in the press).
12. Van Zijl, J. S. V. *Trans. geol. Soc. S. Afr.* **81**, 129–142 (1978).
13. Frick, C. *Trans. geol. Soc. S. Afr.* **88**, 225–243 (1985).
14. Von Gruenewaldt, G., Sharpe, M. R. & Hatton, C. J. *Econ. Geol.* **80**, 803–812 (1985).
15. Clubley-Armstrong, A. R. & Sharpe, M. R. *Trans. geol. Soc. S. Afr.* **82**, 23–36 (1979).

Late Quaternary alluvial history of the northwestern Deccan upland region

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Continental and oceanic sediments from tropical Africa, Australia and South America have recorded analogous changes in climate and stream behaviour during the late Quaternary¹⁻⁴. Recently, data from north-central India have provided evidence for late Quaternary changes in river regime, in response to global changes in climate in the intertropical zone^{4,5}. Here we report evidence for late Quaternary river metamorphosis⁶ from the northwestern Deccan upland region of India. Radiocarbon dates combined with stratigraphic data have allowed the identification of major episodes of erosion and deposition^{7,8}. Supporting evidence from the Arabian sea^{2,3} demonstrates that the successive phases of aggradation and incision are linked to late Quaternary climatic changes^{7,8}.

The landscape of the northwestern Deccan upland region is dominantly erosional and there are no known fossiliferous fluvial or lacustrine sediments of early Quaternary or Tertiary age. The Godavari, Bhima and Krishna rivers originate in the elevated high rainfall zone of the Western Ghats (700–1,400 m above sea level (ASL)) and flow through broad valleys towards the semi-arid east (Fig. 1a). The area is covered by Deccan traps (basalts) of Cretaceous–Eocene age. Rainfall distribution is uneven and more than two-thirds of the drainage area is in the rain shadow of the Western Ghats. The rivers are allocthonous and the peak discharge coincides with the south-west monsoon. The upland rivers are incised 5–20 m into their late Quaternary flood plains. Valley cross-sections show two alluvial fills and two river terraces⁷ (Fig. 1b). The fluvial deposits are $< 5 \text{ km}$ wide, discontinuous and generally unfossiliferous^{7,8}. Stratigraphic exposures along the major rivers and borehole data have allowed us to reconstruct the late Quaternary alluvial history of the area investigated.

Two generations of fluvial deposit with quite different characteristics are apparent. The older deposits termed the 'Upper

Bhima formation'⁹ (UBF) are 5–35 m thick and comprise sandy-pebbly-cobbly gravel at the base, resting unconformably on basalts (Fig. 2a). The gravel is rich in rounded to sub-rounded clasts of basalts, quartz and chaledony, and is cemented by calcium carbonate. The gravel is crudely bedded, clast-supported and represents near-channel deposits⁷. This litho-unit is disconformably overlain by yellow brown or reddish brown sandy silts (silt + clay = 40–60%). Lenticular patches of sands and sandy-pebbly gravel intergrade with the silts in the lower part of the unit (Fig. 2a). In general, the silts and loams are laminated to massive, while planar cross-stratification characterize coarser beds. Calcareous bands of groundwater origin and nodules and tubules of pedogenic origin, are common in this formation. Fissured clays varying in thickness from 0.2 to 5.0 m also occur as channel pool deposits⁷ in association with the gravel and silt. The uppermost sandy-silty unit (2–10 m thick) is generally massive and contains lenses of channel sand and colluvial gravel. The younger formation (2–15 m thick), inset against the older one and designated as 'post-black soil formation'⁹ (PBF), is represented by non-calcareous dark brown sandy-silts, with gravelly lenses at the base. This formation exhibits fine alternations between sands and silts and represents overbank accretion⁷ (Fig. 2b). Texturally, the PBF is finer than the UBF. Laboratory studies of the finer sediments of both PBF and UBF show that these sediments are alkaline (pH 7–9) and are rich in minerals like plagioclase, augite, magnetite and ilmenite. The clay fraction is dominated by montmorillonite and minerals of the beidellite group⁸ and the fine sediments of the PBF appear to be redeposited UBF sandy silts.

The UBF and the PBF, although generally lacking fossils, have yielded animal and plant fossils and abundant archaeological material⁷⁻¹⁰. The basal coarse member of the UBF contains Acheulian and middle Palaeolithic artefacts, wood, and animal fossils (Fig. 2a)⁸⁻¹⁰. Faunal remains of *Bos* species *Elephas* species and *Hippopotamus* species have been found in this litho-unit⁸⁻¹⁰. The upper fine member of this formation has yielded Upper Palaeolithic and Epi-palaeolithic artefacts and fossilized animal bones and wood⁸⁻¹⁰. The PBF in comparison, has yielded drift wood and bones (animal and human), potsherds and stone artefacts of Neolithic–Chalcolithic age⁸⁻¹⁰.

Radiocarbon dates from different sites within the study area (Fig. 1a)^{7,11,12} provide some indications of the probable age of the alluvial deposits (Table 1). Relative dates, based on fluorine-

Table 1 Carbon-14 dates for charcoal, wood, shells and bone from the upland region of northwestern Deccan (India)

	Rivers	Lab. No.	Material	¹⁴ C date yr BP
PBF	Ghod	TF-1330	wood	3,175 ± 105
	Godavari	PRL-77	charcoal	3,400 ± 115
	Godavari	PRL-384	charcoal	3,630 ± 100
	Krishna	TF-1213	shells	3,855 ± 710
	Sina	BS-458*	wood	4,260 ± 110
	Nandi	BS-417	shells	7,150 ± 80
UBF u.m.	Krishna	TF-1178	shells	10,035 ± 125
	Mula	TF-1111	bone	10,310 ± 155
	Ghod	BS-146	shells	11,700 ± 150
	Pravara	BS-576	shells	12,850 ± 190
	Pravara	PRL-470	shells	12,890 ± 350
	Pravara	BS-575	shells	16,420 ± 260
	Godavari	TF-891†	shells	17,075 ± 660
	Ghod	TF-1177	shells	19,290 ± 360
	Ghod	TF-1003	shells	21,725 ⁺⁶³⁰ ₋₅₈₅
	Ghod	TF-1003	shells	21,725 ⁺⁶³⁰ ₋₅₈₅
UBF l.m.	Agran	BS-661	shells	21,780 ± 460
	Pravara	BS-78	shells	24,670 ± 170
	Godavari	BS-163	shells	26,635 ± 425
	Godavari	BS-662	shells	28,390 ± 980
	Mula	TF-345	wood	30,030 ± 5715
	Krishna	TF-1004	shells	38,480 ⁺⁸⁹⁴⁰ ₋₄₁₂₅
	Mula	TF-217	wood	>39,000
	Ghod	BS-43	wood	39,010 ± 3215

Remaining dates after Deccan College, Pune. u.m., Upper fine member; l.m., lower coarse member.

* ref. 11; † ref. 12.

phosphate ratio (100 F/P₂O₅)¹³ on over a dozen bones, support the radiocarbon dates. Much of the exposed UBF belongs to the late Pleistocene including last glacial maximum, and ranges in age from ~>30,000 to ~10,000 yr BP. The basal gravel of the UBF is beyond the range of C-14 dating. However, the occurrence of early and late Acheulian artefact assemblages in this unit suggests a late-middle Pleistocene age⁷. Fluorine-phosphate dating of the associated fossil fauna has given values ranging between 6 and 8 which are comparable with the late-middle Pleistocene bones from Narmada and other parts of the Deccan¹³. The younger formation (PBF) is of late Holocene age (~4,500 to ~3,000 yr BP). The Neolithic-Chalcolithic cultural remains and relative dates on bone further support this inferred age.

Periods of aggradation and degradation during the late Quaternary are immediately recognizable from Table 1. It appears that near-equilibrium conditions have prevailed for only limited periods of time during the last ~>30,000 yr. Major aggradation occurred between ~>30,000 and 10,000 yr BP, with deposition of fine sediments between ~17,000 and 10,000 yr BP, coevally with colluvial sediments and marginal aeolian deposition of loess in the foothills, away from the banks¹⁴. Incision took place between ~10,000 and 4,500 yr BP, followed by deposition of a lower magnitude, from ~4,500 to ~3,000 yr BP. Since then, incision with intermittent deposition have dominated the fluvial regime^{7,14}.

On the basis of Schumm's¹⁵ classification of alluvial channels and the modern channel data from the upland region, two distinct channel-fill types can be recognised within the deposits, namely, bedload and suspended load channels. The suspended load channels, dominated by silt-clay deposits, were narrow and in most cases sinuous^{7,14}. Lateral migration of these meandering channels up to 300 m during the end-Pleistocene is clearly demonstrated by the borehole data¹⁴. The coarse member, in contrast, reflects deposition by shallow, wide, non-meandering, seasonally high discharge bed-load streams^{7,14}. The basal coarse member of the UBF is overlain by overbank deposits, indicating river transformation (metamorphosis) from bedload streams

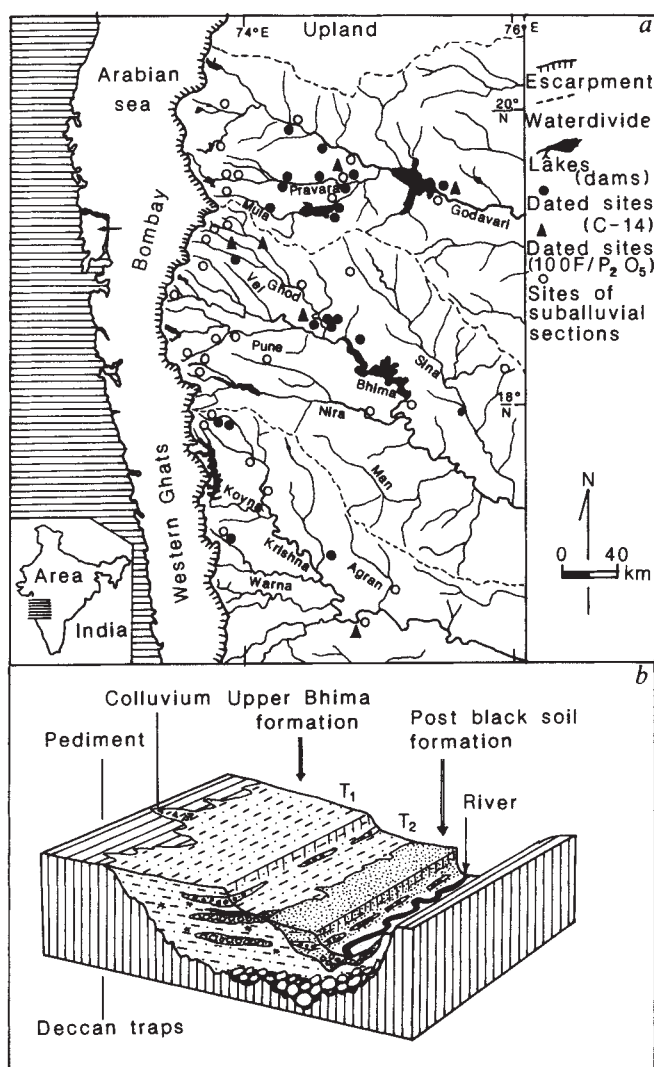


Fig. 1 Map of the area investigated (a); generalized block diagram showing the two-alluvial-fill and two-terrace combination in valley alluvium (b), T₁ = terrace 1 and T₂ = terrace 2 (not to scale).

(~>30,000–17,000 yr BP) to suspended load channels during the closing phase of the Pleistocene. The textural and stratigraphical properties of the PBF denote over-bank and near-bank accretion of fine sediments under conditions of low current velocity and reduced runoff^{7,14}. The early and late Holocene rejuvenation, incision and terrace formation imply increased stream energy and reduced sediment load^{7,14}.

The tentative periods of incision and deposition recognized for the uplands rivers, are consistent with the regional climatic changes of the late Quaternary, as evidenced from micro-fossil, oxygen isotope and sediment studies of deep sea cores and surface sediments from the northern Arabian sea^{2,3,16,17}, and from geoarchaeological studies in western India^{8–10}. The late Pleistocene aggradational phase coincided with the weakening of the south-west monsoon, particularly during last glacial maximum (~18,000 yr BP) and reversal of this pattern during early Holocene increased runoff and the rivers incised. Comparison of the late Quaternary behaviour of these east flowing rivers with the sea-level changes along the west coast of India, reveals an inverse correlation between the two¹⁸. During low sea-level, the sea receded by >200 km and increased the continentality and aridity in the upland region, leading to aggradation (Fig. 3a). In contrast, high sea-level reduced the continentality and increased runoff, and the rivers incised vigorously¹⁸.

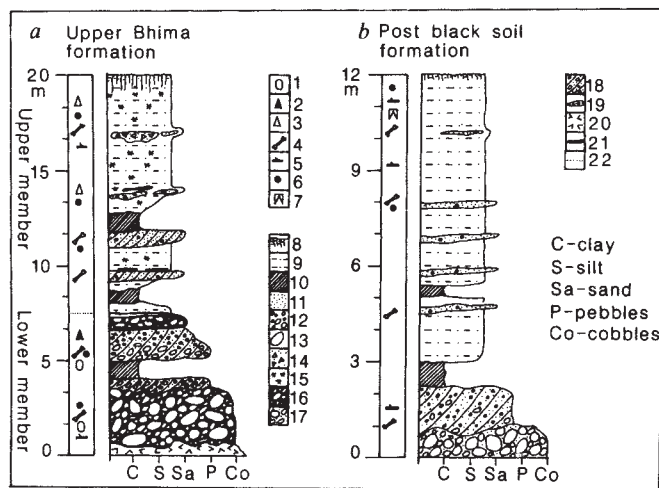


Fig. 2 Composite lithosections for the UBF (a) and PBF (b); 1, Acheulian, 2, Middle Palaeolithic, 3, Upper and Epi-palaeolithic artefacts, 4, fossil bones, 5, fossil wood, 6, shells, 7, Chalcolithic artefacts; 8, soil, 9, sandy-silt, 10, fissured clays, 11, sand, 12, pebbly gravel, 13, pebbly-cobbly gravel, 14, colluvial gravel, 15, calcrete, 16, indurated gravel, 17, unconsolidated gravel, 18, cross-bedding, 19, lenses, 20, basalt, 21, bedded calcrete, 22, erosional surface.

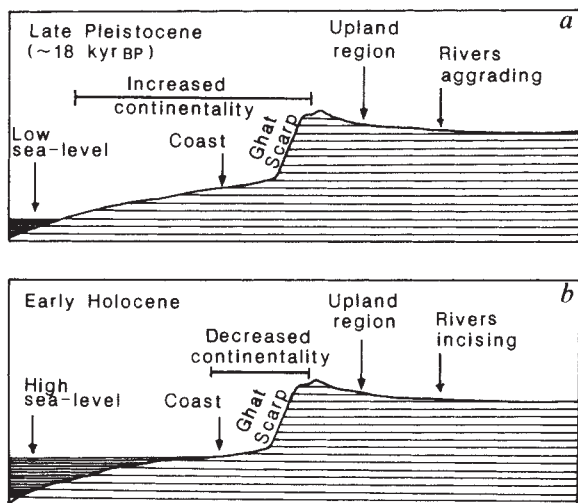


Fig. 3 Relation between the sea-level changes along the west coast of India and the nature of fluvial regimes in the upland region of northwestern Deccan between ~30,000 and 4,500 yr BP (not to scale).

(Fig. 3b). Aggradation, thus, occurred during low sea-level and intense incision followed the early Holocene rapid rise in the sea-level (Fig. 3). The late Holocene changes in climate were comparatively minor and therefore do not correlate with the sea-level changes along the west coast of India.

Aridity in the study area is also evident from the thick (2–8 m) end-Pleistocene colluvial deposits in the foothills of many valleys¹⁴. A drier climate during the late Holocene is shown by pedocalcic soils occurring between the Chalcolithic and the early historical deposits and by changes in the dietary habits of Chalcolithic inhabitants from cultivated cereals to animal food¹⁹. The downfall of the Chalcolithic cultures close to one millennium BC has also been attributed to severe droughts in the western and central India¹⁹. Archaeological evidence in the form of cultural growth and the economic development of an early agricultural community, suggest amelioration in climate between 2,200 and 1,500 yr BP⁷.

The changes in the fluvial deposition and downcutting and the environments deduced for the upland region of northwestern Deccan are similar to those of the Son and Belan in north-central India^{4,5}, the Nile²⁰, the Amazon²¹ and northern Chile²²; and coincide with global changes in climate in the intertropical zone^{1,4}.

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- Williams, M. A. J. in *Environmental Changes and Tropical Geomorphology* (eds Douglas, I. & Spencer, T.) 219–233 (Allen & Unwin, London, 1985).
- Duplessy, J. C. *Nature* **295**, 494–498 (1982).
- Van Compo, E., Duplessy, J. C. & Rossignol-Strick, M. *Nature* **296**, 56–59 (1982).
- Williams, M. A. J. & Royce, K. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **38**, 139–162 (1982).
- Williams, M. A. J. & Clarke, M. F. *Nature* **308**, 633–635 (1984).
- Schumm, S. A. *The Fluvial System*, (Wiley, New York, 1977).
- Rajaguru, S. N. & Kale, V. S. *J. geol. Soc. Ind.* **26**, 16–27 (1985).
- Corvinus, G., Rajaguru, S. N. & Mujumdar, G. G. *Quartar* **23–24**, 53–69 (1973).
- Kajale, M. D., Badam, G. L. & Rajaguru, S. N. *Geophytology* **6**, 122–132 (1976).
- Sankalia, H. D. *Prehistory and Protohistory of India and Pakistan* (Deccan College, Pune, 1974).
- Tandale, T. D. *Current Science*, **54**, 1280–1281 (1985).
- Rajamani, V. & Parthasarathy, A. in *Recent Researches in Geology*, **9** (ed. Merth, S. S.) 252–260 (Hindustan, New Delhi, 1982).
- Joshi, R. V. & Kshirsagar, A. A. *Fluorine and Other Chemical Studies of Quaternary Animal Fossils of India* (Deccan College, Pune, 1986).
- Kale, V. S. & Rajaguru, S. N. *Ann. NAGI*, **5**, 1–9 (1985).
- Schumm, S. A. *Rev. Earth planet. Sci.* **13**, 5–27 (1985).
- Fontugne, M. R. & Duplessy, J. C. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **56**, 59–88 (1986).
- Nair, R. R. & Hashimi, N. H. *Proc. Ind. Acad. Sci.* **89B**, 299–315 (1980).
- Kale, V. S. & Rajaguru, S. N. *Bull. Deccan Coll. Res. Inst.* **45**, 68–70 (1986).
- Dhavalikar, M. K. in *Recent Advances in Indian Archaeology* (eds Deo, S. B. & Paddayya, K.) 65–73 (Deccan College, Pune, 1985).
- Adamson, D. A., Gasse, F., Street, F. A. & Williams, M. A. J. *Nature* **288**, 50–54 (1980).
- Damuth, J. E. & Fairbridge, R. W. *Bull. Geol. Soc. Am.* **81**, 189–206 (1970).
- Lynch, T. F. *Geoarchaeology* **1**, 145–162 (1986).

Identification of an aphid sex pheromone

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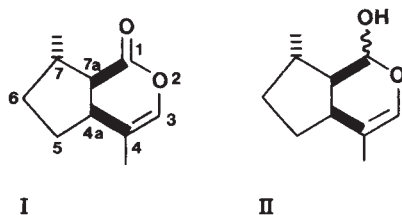
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Although aphids reproduce asexually on their host plants during the summer, many species migrate to a winter host where sexual reproduction occurs. Males of certain species locate mates by means of a sex attractant pheromone released from the hind legs of the females^{1–4}. Here we report the identification of such a pheromone from the vetch aphid *Megoura viciae*. Extracts of the excised legs of sexual females were analysed by gas chromatography-coupled single cell recording techniques (GC-SCR)⁵ and by gas chromatography-coupled mass spectrometry (GC-MS). A tentative identification by MS analysis of the two active components as specific isomers of the monoterpenoids nepetalactone (I), a known cat attractant, and nepetalactol (II) was confirmed by comparison with authenticated compounds isolated or synthesized from natural products. Although neither compound was attractive to males when presented alone in the laboratory bioassay, a mixture of the two components produced a response equal to that elicited by the female leg extract. Thus the sex pheromone of *M. viciae* comprises a synergistic mixture of the nepetalactone I and the nepetalactol II. The identification of aphid sex pheromones will provide a further means of investigating and manipulating the behaviour of these ubiquitous pests.

Perception of the sex pheromone in aphids is thought to be associated with the secondary rhinaria on the antennae of males⁴. With electrophysiological recording techniques in which single-cell preparations are used to monitor the effluent from high-resolution gas chromatography (GC-SCR)⁵ we have identified the sex pheromone of an aphid. Viviparae of *Megoura*

viciae were maintained on bean plants, *Vicia faba*, at 15 °C in a 12 h day/night light regime in order to produce sexual forms. The oviparous females obtained were anaesthetized with ether and the hind legs removed and extracted with pentane. This extract was behaviourally active in a laboratory bioassay (Table 1). The bioassay was done in a simple choice chamber consisting of a 15 cm Petri dish, in the base of which were two holes covered with a layer of muslin. Under these holes were placed either the treatment, applied to a filter paper square, or an empty pot as a control. Ten male *M. viciae* were placed in the dish and numbers of aphids positioned over each hole (the 'test area') were recorded once a minute for 10 minutes. During the test, the dish was placed in a box, illuminated only from above, which was aired with an electric fan between tests. Female leg extract (10 µl) was used at a concentration equivalent to 1 leg per µl of solvent. Pheromone components were tested at a similar concentration, 10 µl being applied when tested alone, and 5 µl of each when tested together.

Single-cell recordings showed that olfactory cells associated with the secondary rhinaria of males responded to the female leg extract (Fig. 1). GC-SCR, using these recordings to monitor the column effluent, demonstrated the presence of two electrophysiologically active components. GC-coupled mass spectrometry (GC-MS) (70 eV, 200 °C on a VG 70-250 mass spectrometer with an OV-101 bonded phase fused silica capillary column) showed that their relative molecular masses (M_r) were 168 (compound X) and 166 (compound Y). The fragmentation of compound Y was similar to that of nepetalactone, which has three known diastereoisomers⁶. These were isolated from catmint plants, *Nepeta cataria* and *N. mussinii*, by HPLC (50 × 1 cm



column, 10 µ silica, Partisil Magnum 9 10/50, 5% (v/v) ether/hexane, monitoring at 208 nm) and structures were confirmed by ¹³C and ¹H nuclear magnetic resonance (NMR) spectroscopy. The three diastereoisomers were well resolved on chromatography: (4aS, 7S, 7aR)-nepetalactone (I) from *N. cataria* had the same GC retention time as compound Y and the mass spectrum⁶, when run under the same conditions, was identical to that from the aphid, m/z 166 (M^+ , 61%), 151 (7), 138 (19), 123 (70), 109 (41), 95 (59), 81 (81), 69 (100), 67 (58), 55(35), 41 (74), 39 (53). In addition, the peak from compound Y in the aphid extract was enhanced by co-injection with the plant-derived nepetalactone I on the OV-101 and on a bonded Carbowax 20-M fused silica capillary column. The (4aS, 7S, 7aR)-nepetalactone was active electrophysiologically (Fig. 1), but gave only a weak response in the behavioural bioassay (Table 1). The enantiomer (4aR, 7R, 7aS)-nepetalactone was prepared from (*R*)-pulegone by a route based on Wolinsky's work⁷ but showed no electrophysiological activity (Fig. 1). It was concluded that compound Y was (4aS, 7S, 7aR)-nepetalactone (I) but that for behavioural activity, compound X was required.

The mass spectrum for compound X, m/z 168 (M^+ , 45%), 135 (52), 97 (57), 84 (70), 81 (72), 71 (77), 67 (61), 58 (58), 55 (52), 43 (60), 41 (100), 39 (52), did not closely resemble any published spectrum. However, iridodial has M_r 168 and gives, by McLafferty rearrangement, an abundant ion fragment at m/z 58. This compound was obtained from the devil's coach horse, *Staphylinus (Ocypus) olens*^{8,9}, but chromatographed before compound X and gave a very different mass spectrum. It was considered likely that compound X was a reduction product of

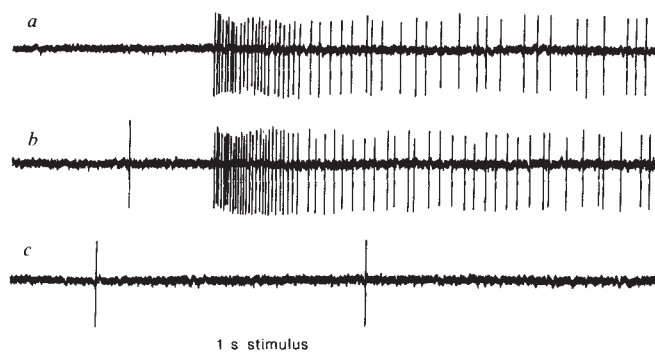


Fig. 1 Responses of a male *M. viciae* olfactory cell to stimulation with ~20 leg equivalents of *a*, female leg extract; *b*, nepetalactone I; *c*, enantiomer of nepetalactone I.

the second component Y and the conversion of (4aS, 7S, 7aR)-nepetalactone into compound X was attempted. This was achieved by reduction with diisobutylaluminium hydride. The product gave a single peak on GC which was enhanced when co-injected with the aphid extract on both the OV-101 and Carbowax 20-M capillary columns and gave a mass spectrum which was similar to that obtained for the aphid compound X. The nepetalactol structure (II) was established for the reduction product by ¹³C NMR: peaks at 93.9 p.p.m. (–OCHOH), 133.5 and 113.1 (C=CH–O) and 7 others upfield, and by ¹H NMR: characteristic peaks at 6.01 p.p.m. (=CH–O), 4.85 (O–CH–O) and 2.82 (OH), with 5 Hz couplings from the 4.85 peak to both the OH peak and an octet at 1.64 (H-7a). The absolute stereochemistry of the parent compound, vis (4aS, 7S, 7aR), was retained. Although reduction to the lactol (II) created a new asymmetric centre at carbon-1, only one diastereoisomer was obtained. The 5 Hz coupling between Hs 1 and 7a in the NMR spectrum was not, without further study, sufficient to determine unequivocally the absolute stereochemistry at C-1. The lactol structure was claimed as a rearrangement product of iridodial but was not fully characterized^{8,10}.

The (4aS, 7S, 7aR)-nepetalactol was electrophysiologically active but showed no behavioural activity when tested alone in the laboratory bioassay. However, when applied together with the (4aS, 7S, 7aR)-nepetalactone, male response was equal to that elicited by a female leg extract containing similar amounts of each component, ~0.5–1 ng per leg (Table 1). Thus the sex pheromone of the aphid *Megoura viciae* comprises the nepetalactone I and the nepetalactol II. The nature of the sex pheromones of other aphids is now under investigation: initial studies have shown that both the black bean aphid, *Aphis fabae* Scopoli, and the pea aphid, *Acyrtosiphon pisum* (Harris), have olfactory receptors for these two compounds. The existence of intra- and interspecific responses to aphid sex pheromones^{1,2,4} suggests a lack of specificity that may prove advantageous to their use for aphid control.

The presence of nepetalactone in certain plants is unlikely to be directly associated with the composition of the aphid sex pheromone and may be the result of an economy of biosynthetic routes in the plant and animal kingdoms. Compounds closely

Table 1 Behavioural bioassay with male *M. viciae*

Treatment	No. of replicates	Mean aphid score		<i>P</i>
		Treated	Control	
Leg extract	12	5.7 ± 0.38	0.08 ± 0.08	<0.001
Nepetalactone I	4	1.75 ± 0.25	0.5 ± 0.28	NS
Nepetalactol II	4	0.66 ± 0.66	0.66 ± 0.33	NS
I + II	12	5.75 ± 0.41	0	<0.001
No treatment	4	0.5 ± 0.5	0.5 ± 0.29	NS

Aphid score, maximum no. of aphids observed in test area; NS, not significant at probability level 0.05.

related to both the nepetalactone I and the nepetalactol II have been shown to influence the behaviour of certain aphid predators¹¹ and it is possible that the sex pheromone may act as a kairomone in attracting predatory species. However, although the nepetalactone I is also known to be a powerful attractant for cats and other Felidae¹², a behavioural link in this case would be hard to conceive.

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- Pettersen, J. *Entomol. Scand.* **1**, 63-73 (1970).
- Pettersen, J. *Entomol. Scand.* **2**, 81-93 (1971).
- Marsh, D. *Nature new Biol.* **238**, 31-32 (1972).
- Marsh, D. *J. Ent.* **50**, 43-64 (1975).
- Wadhams, L. J. *Z. Naturf.* **37C**, 947-952 (1982).
- Eisenbraun, E. J. *et al. J. org. Chem.* **45**, 3811-3814 (1980).
- Wolinsky, J. & Eustace, E. J. *J. org. Chem.* **37**, 3376-3378 (1972).
- Abou-Donia, S. A., Fish, L. J. & Pattenden, G. *Tetrahedron Lett.* **43**, 4037-4038 (1971).
- Fish, L. J. & Pattenden, G. *J. Insect Physiol.* **21**, 741-744 (1975).
- Cavill, G. W. K. & Ford, D. L. *Aust. J. Chem.* **13**, 296-310 (1960).
- Sakan, T., Ise, S. & Hyeon, S. B. in *Control of Insect Behaviour by Natural Products* (ed. Wood, D. L., Silverstein, R. M. & Nakajima, M.) 237-247 (Academic, New York, 1970).
- McElvain, S. M., Walters, P. M. & Bright, R. D. *J. Am. chem. Soc.* **64**, 1828-1831 (1942).

An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*

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Blooms of the toxic dinoflagellate *Gonyaulax tamarensis* (synonyms *Protogonyaulax tamarensis*¹ and *Alexandrium tamarensis*²) cause outbreaks of paralytic shellfish poisoning (PSP) in coastal waters throughout the world. In the Gulf of Maine, episodes occur between April and November, a seasonality due in part to life-cycle alternations between motile, vegetative cells and resting cysts which overwinter in bottom sediments^{3,4}. Newly formed cysts have a mandatory 2-6 month dormancy period during which germination is not possible⁵, but once mature, the resting state will continue if temperatures are unfavourable⁵ or oxygen is unavailable⁶. We now report another factor controlling germination of cysts of *G. tamarensis* from deep coastal waters—an endogenous annual clock that can override an otherwise favourable environment for germination. Similar annual variability in germination has not been observed for cysts of this species from shallow estuaries. These results represent the first conclusive demonstration of an endogenous circannual rhythm in a marine plant. They are evolutionarily and ecologically significant because an endogenous annual clock can lead to the release of motile cells into deep and relatively invariant bottom waters at those times when temperature and light at the surface are suitable for growth. In shallow waters where seasonal variability is large and extends to bottom sediments, a strategy similar to that of the seeds of terrestrial plants would be more appropriate, namely a direct coupling between germination and the external environment.

The annual rhythm was first observed in cysts from freshly collected sediment cores. Regardless of depth of burial within the sediment or overlying water depth, germination frequency of cysts from the Gulf of Maine oscillated systematically during the two-year study (Fig. 1b). Cysts of other species from the Gulf of Maine and of the same species from a shallow Cape Cod estuary had high germination frequencies in the same tissue culture plates and medium.

The germination rhythm was also evident in monthly subsamples from an August core stored in the dark at 2 °C (Fig. 1c).

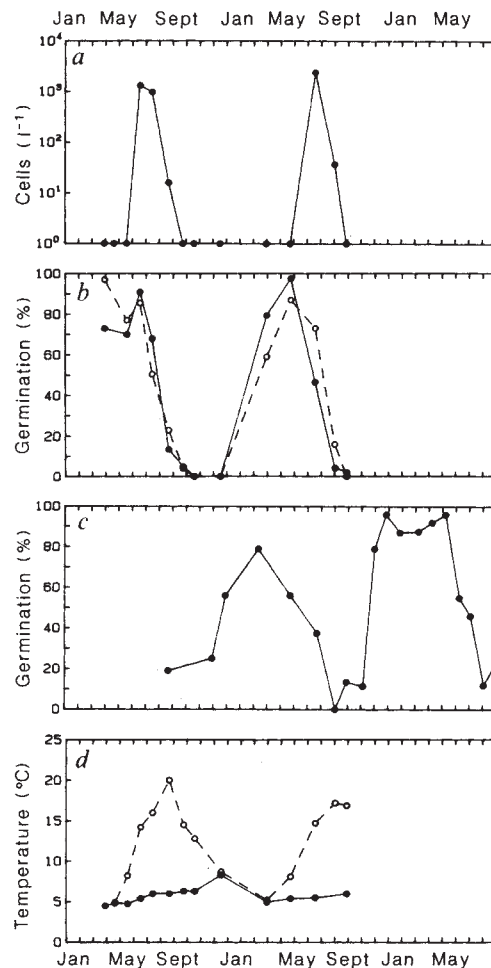


Fig. 1 Population dynamics of cysts and motile cells of *Gonyaulax tamarensis*. **a**, Average motile cell concentration in the top 30 m of the water column from continuously pumped samples at Station 29 (160 m deep, 43° N 70°19' W) and Station 30 (60 m deep, 43°01' N, 70°33' W) in the Gulf of Maine. **b**, Average germination frequency of cysts isolated from (●) top 2 cm and (○) 5- and 6-cm depths in freshly collected cores from Station 29 and Station 30. **c**, Germination frequency of cysts isolated from sediment stored in the laboratory under constant conditions. Sediment from the 3-6-cm depth interval of cores from Station 29 on 17 August 1984. **d**, Bottom (●) and surface (○) water temperature at Station 29. **Methods.** Cores were extruded on board ship, the outer circumference discarded, and the central portion of each depth interval stored in the dark at the temperature of the bottom water during transit to the laboratory. Within 48 h, the samples were sonicated, sieved¹⁷, and the 20-80-μm fraction resuspended in germination medium. To maintain constant germination conditions, all cyst suspensions and germination medium used sea water from the same two polyethylene carboys of Sargasso Sea water, filtered through a 0.2-μm filter and diluted 10% with distilled, deionized water. This was considered more consistent in composition and more conducive to germination than artificial sea water. Germination medium was 'f/2' of Guillard and Ryther¹⁸. Each data point in **b** and **c** represents the germination success of at least 48 cysts that were isolated and placed individually in separate wells of a 96-well tissue culture plate in 130 μl of medium. Plates were taped to minimize evaporation and incubated on a 14:10 h light to dark photoperiod at 15 °C and 150 μE m⁻² s⁻¹ (cool-white fluorescent light) for one month. Excystment was successful if the germling cell was able to emerge completely from the cyst wall. The germination data in **c** were obtained using small aliquots removed from the central portion of a large sediment sample collected in August 1984 from Station 29. Subsamples were processed and cysts isolated and incubated as described above. The bulk mud sample was stored in the dark at 2 °C.

Thus the reduced germination success from August to December is not due to the primary dormancy of immature cysts; maturation would be evidenced by an increasing trend in germination frequency through time, remaining constant and high thereafter. Persistence of the germination rhythm for two years under constant storage conditions indicates endogenous control.

Annual cycles have long been recognized in plants and animals⁷, but many of the rhythms occur in response to external cues and disappear with storage in constant conditions. True endogenous control of annual rhythms is most easily demonstrated in animals⁸ and is difficult to document in plants⁹. The germination of some plant seeds can vary over an annual cycle, in that seeds can be alternately responsive and unresponsive to the same germination conditions^{10,11}, but this rhythm typically disappears with storage in constant conditions. The phenomenon is thus termed secondary dormancy—the environmentally mediated re-induction of a state of deep dormancy. The germination rhythm in *G. tamarensis* cysts isolated from monthly core samples (Fig. 1b) could be attributed to secondary dormancy as the 'storage' environment could not be controlled. However, persistence of the rhythm for two years under constant conditions in the laboratory (Fig. 1c) is consistent with endogenous regulation.

Suggestions of annual rhythms are found in three studies of *G. tamarensis*. Yentsch and Mague¹² report evidence suggestive of an annual cycle in maximum growth rate and cell yield. The data are not conclusive, however, as they were compiled from the control cultures of a series of experiments conducted in different growth chambers by several investigators. Dale *et al.*¹³ and Fukuyo *et al.*¹⁴ could not germinate *G. tamarensis* cysts from Gulf of Maine and Ofunato Bay sediments during the fall months. Both studies attributed the lack of germination to immature cysts, but it is also possible that both used cysts in the unresponsive phase of their annual cycle. Interestingly, germination of the cysts of a closely related strain (or species^{1,2}) of *Gonyaulax* did not follow a rhythmic pattern during the Ofunato Bay study¹⁴. Similarly, during this study and others¹⁵, we observed high germination frequencies for cysts of *G. tamarensis* from a shallow estuary throughout the year. This implies that the annual rhythm is maintained only in certain populations or strains of this organism.

Control of cyst germination by an endogenous clock would be most useful in deep coastal waters where bottom temperatures are relatively invariant and photoperiodic cues non-existent. Without endogenous regulation, cyst germination would occur throughout the year, because bottom temperatures in the Gulf of Maine remain within a permissive 4–6 °C range (Fig. 1d). Instead, we observe a cyst germination rhythm with the same seasonality as the motile cell blooms (Fig. 1a). If further studies verify that there is no similar timing mechanism in shallow water strains of *G. tamarensis*, an environmental separation of genotypes is implied. This would not be surprising as a genetic basis for circadian rhythms has been reported in the fruit fly *Drosophila*¹⁶. Shallow water sediments experience considerable environmental variability from year to year, so inflexible endogenous control of germination might be a disadvantage compared to a strategy whereby germination is directly controlled by the environment. Shallow water populations of *G. tamarensis* may thus be comparable to terrestrial plants whose seeds are responsive only to the external environment for the breaking of dormancy.

More work is needed to explain the observed annual rhythm and to understand differences between strains or populations. If endogenous annual rhythms are found in the germination patterns of other cyst-forming dinoflagellates or in the growth or metabolism of phytoplankton in general, such information would have profound implications with respect to bloom dynamics and the temporal succession of algal species.

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1. Taylor, F. J. R. in *Toxic Dinoflagellates* (eds Anderson, D. M., White, A. W. & Baden, D. G.) 11–26 (Elsevier, New York, 1985).
2. Balech, E. in *Toxic Dinoflagellates* (eds Anderson, D. M., White, A. W. & Baden, D. G.) 33–38 (Elsevier, New York, 1985).
3. Dale, B. *Sarsia* **63**, 29–34 (1977).
4. Anderson, D. M. & Wall, D. J. *Phycol.* **14**, 224–234 (1978).
5. Anderson, D. M. *J. Phycol.* **16**, 166–172 (1980).
6. Anderson, D. M., Taylor, C. D. & Armbrust, E. V. *Limnol. Oceanogr.* (in the press).
7. Farmer, D. S. A. *Rev. Physiol.* **47**, 65–82 (1985).
8. Kenagy, G. J. *Comp. Biochem. Physiol.* **134**, 333–340 (1981).
9. Sweeney, B. M. *Rhythmic Phenomena in Plants* (Academic, New York, 1969).
10. Karssen, C. M. *Israel J. Bot.* **29**, 45–64 (1981).
11. Bewley, J. D. & Black, M. *Physiology and Biochemistry of Seeds in Relation to Germination* Vol. 2 (Springer, Berlin, 1982).
12. Yentsch, C. M. & Mague, F. C. *Int. J. Chronobiol.* **7**, 77–84 (1980).
13. Dale, B., Yentsch, C. M. & Hurst, J. W. *Science* **201**, 1223–1225 (1978).
14. Fukuyo, Y., Watanabe, M. M. & Watanabe, M. in *Eutrophication and Red Tides in the Coastal Marine Environment* 43–52 (National Institute (Japan) for Environmental Studies, Tsukuba, 1982).
15. Anderson, D. M. & Morel, F. M. M. *Estuar. coast. mar. Sci.* **8**, 279–293 (1979).
16. Konopka, R. J. & Benzer, S. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2112 (1971).
17. Wall, D. & Dale, B. *Micropaleontology* **14**, 265–304 (1968).
18. Guillard, R. R. L. & Ryther, J. H. *Can. J. Microbiol.* **8**, 229–239 (1962).

Transient cells of the developing mammalian telencephalon are peptide-immunoreactive neurons

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In the development of the mammalian telencephalon, the genesis of neurons destined for the various layers of the cerebral cortex is preceded by the generation of a population of cells that comes to reside in the subplate and marginal zones¹ (see ref. 2 for nomenclature). In the cat, these cells are present in large numbers during development, when their location is correlated with the arrival and accumulation of ingrowing axonal systems^{3–6} and with synapses^{7–12}. However, as the brain matures, the cells disappear and the white matter and layer 1 of the adult emerge^{1,13,14}. Their disappearance occurs in concert with the invasion of the cortical plate by the axonal systems and with the elimination of the synapses from the subplate^{1,4,7,9,12}. Here we report that the subplate cells have properties typical of mature neurons. They have the ultrastructural appearance of neurons and receive synaptic contacts. They also have long projections and are immunoreactive for MAP2 (microtubule associated protein 2). Further, subpopulations are immunoreactive for one of several neuropeptides. These observations suggest that during the fetal and early postnatal development of the mammalian telencephalon the subplate cells function as neurons in synaptic circuitry that disappears by adulthood.

We identified the subplate and marginal zone cells unambiguously by their birthdate. These cells undergo their final round of cell division and migrate away from the ventricular zone between embryonic day 24 (E24) and E30 (gestation is 65 days in the cat)¹. In contrast, cells of the cortical layers 2–6 are not generated until after E30¹⁵. Accordingly, intrauterine injections of ³H-thymidine (NEN, 50–80 Ci mmol⁻¹) in sterile physiological saline were made between E26 and E28 in anaesthetized fetuses¹. Ten fetuses were then allowed to develop to later fetal or early postnatal (P) ages: E50 (2 animals), E54 (1), E56 (3), E58 (1) and P7 (3). An additional ten animals at comparable stages of development were examined without prior thymidine labelling. Immunohistochemical and/or retrograde labelling studies were performed and then sections labelled with ³H-

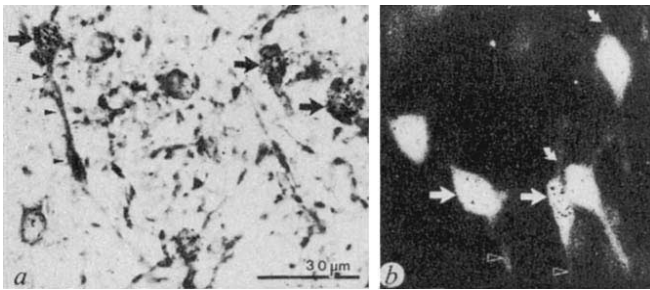


Fig. 1 Subplate cells (*a*) stained with antisera against MAP2, and (*b*) labelled retrogradely following an injection of TRITC into the contralateral hemisphere. Subplate cells were identified by their distinct birthdates with ^3H -thymidine labelling at E27. Cells shown in the photographs of each panel were located deep within the future white matter. In *a*, the brain was prepared for MAP2 immunohistochemistry at E56 by perfusing with 4% paraformaldehyde in 0.1 M sodium phosphate buffer followed by immersion-fixation in a mixture of periodate-lysine-paraformaldehyde³⁴. Cryostat sections (10 μm) were cut and incubated in 1:1000 anti-MAP2¹⁶ or normal rabbit serum. The distribution of specific antibody binding was visualized using peroxidase immunohistochemistry (Vector Labs). Reacted sections were then processed for autoradiography. Arrows, MAP2-immunoreactive cells that are also heavily labelled with ^3H -thymidine, as indicated by the accumulation of silver grains over each nucleus. (Immunoblot analysis indicates that MAP2 is present at E56. See also ref. 30.) A common morphological feature of these cells is the presence of a large dendritic process (arrowheads) directed towards the ventricular surface of the telencephalon (downwards in this figure). In *b*, subplate cells (^3H -thymidine-labelled cells indicated by arrows) are shown to have a long projecting process (probably an axon), as they can be retrogradely labelled following an injection of TRITC deep into the subplate of the opposite hemisphere. Note that processes can extend from the soma either towards the pial surface (curved arrows) and/or towards the ventricular surface (arrowheads). TRITC (Sigma) was injected as a 10% solution in dimethylsulfoxide; multiple injections (0.1 μl) into the contralateral hemisphere were followed by a 48-h survival period. Animals were perfused at P7 (4% paraformaldehyde), and brain sections (80 μm) made on a vibratome were processed for autoradiography and viewed with a fluorescence microscope.

thymidine were autoradiographed to confirm the identity of the early generated cells.

The subplate cells have characteristics of neurons such as immunoreactivity for the neuron-specific protein microtubule-associated protein 2 (MAP2)^{16,17}: every ^3H -thymidine-tagged subplate cell was MAP2 immunoreactive (Fig. 1*a*, arrows). Many of these cells possess a distinctive process often directed towards the ventricular surface (Fig. 1*a*, arrowheads); this and other processes emanating from the soma are frequently beaded. MAP2 is thought to be dendrite-specific at immunohistochemically detectable levels both in adults and during development^{16,17}, so these processes are probably dendrites.

In addition to dendrites, the subplate cells also have long projecting processes, probably axons. This was demonstrated by retrograde labelling experiments in which multiple injections of the fluorescent tracer tetramethylrhodamine isothiocyanate (TRITC) were made deep into the subplate of one hemisphere in eight neonates. Figure 1*b* shows several TRITC-labelled cells in the subplate of the opposite hemisphere that are similar in morphology to the ^3H -thymidine-tagged cells immunoreactive for MAP2. These were found in both the injected as well as the opposite hemisphere. This labelling technique also occasionally revealed processes directed toward the pial surface (Fig. 1*b*, curved arrows; see also Fig. 3*a*). Moreover, some of the TRITC-labelled cells can be autoradiographically labelled by ^3H -thymidine injection made at E27 (Fig. 1*b*, thick arrows), thereby identifying them as subplate cells.

The subplate cells have the ultrastructural appearance of

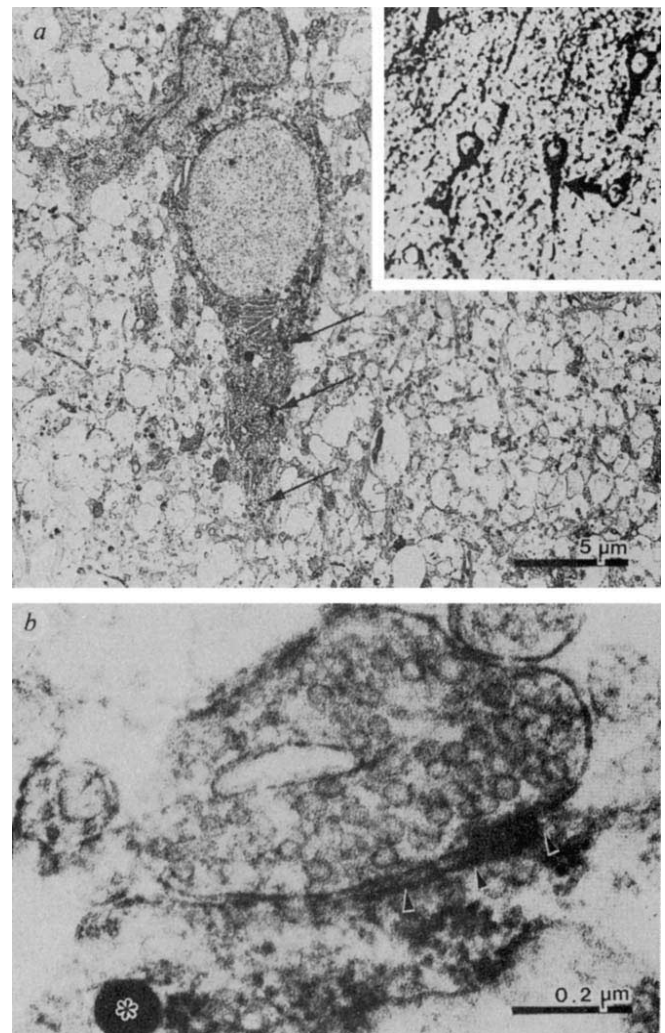
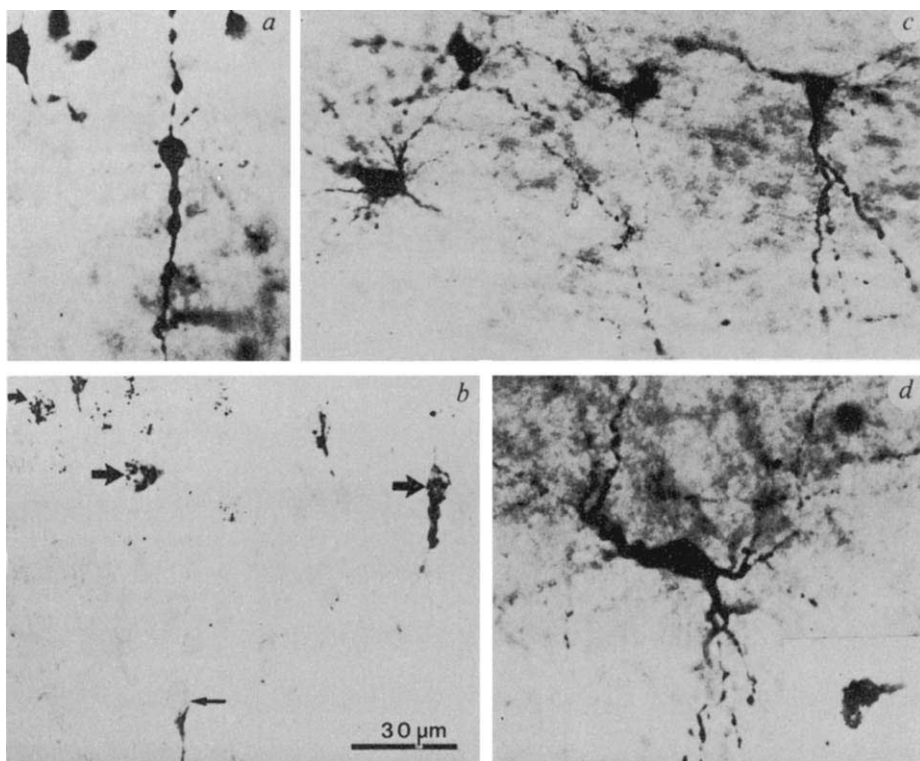


Fig. 2 Ultrastructural evidence showing that subplate cells can receive synapses. Subplate cells were retrogradely labelled in a newborn cat by injecting 40% HRP, 1% lysocleithin in sterile saline, deep into the subplate. The animal was reanaesthetized 24 h later, perfused with mixed aldehydes, and the brain processed for EM-HRP histochemistry using DAB-nickel-cobalt as the chromogen³⁵. In *a*, the inset shows a light micrograph of retrogradely labelled subplate cells (arrow). The pial surface is towards the top of the page. One of the cells in the inset is shown in the electron micrograph. The arrows indicate electron dense, HRP inclusion bodies typical of retrogradely labelled cells. *b*, Higher magnification view of a portion of the distinctive process of a subplate cell identified by the presence of an HRP inclusion body (asterisk). Note the synapse (arrowheads) and the presence of ribosomes that identify the postsynaptic profile as a dendrite.

neurons. Figure 2 shows an electron micrograph of a subplate cell retrogradely labelled with horseradish peroxidase (HRP). Electron-dense inclusion bodies characteristic of retrogradely transported HRP are located within the distinctive processes (Fig. 2*a*, thin arrows), which at higher magnification (Fig. 2*b*) have the morphological characteristics of dendrites including ribosomes and the presence of synaptic contacts. Thus, a variety of different criteria indicate that the subplate cells are neurons.

To characterize these neurons further, their immunoreactivities for several neuropeptides were examined, an approach motivated in part by recent observations that the few neurons embedded in the white matter of adult mammalian neocortex are frequently immunoreactive for neuropeptides¹⁸⁻²². As previous ^3H -thymidine birthdating studies have shown that <10% of the subplate neurons persist into adulthood, where they are

Fig. 3 Subplate cells immunoreactive for the neuropeptide (a, b) SRIF, (c) NPY and (d) CCK. In each case, fetuses received an injection of ^3H -thymidine at E27 to label the subplate cells and at later ages, the brains were prepared for immunohistochemistry as described for Fig. 1. All antisera were used at a dilution of 1:1,000 (SRIF and CCK, Immunonuclear Corporation; NPY, gift from Dr C. Evans, Stanford University). The pattern of peptide immunostaining was specific as it was not present in normal rabbit serum controls (1:1,000) and could be blocked routinely by incubating 50 nM of the appropriate peptide (Peninsula Labs) simultaneously with diluted antisera. The true TRQRYamide carboxy terminus, against which the NPY antiserum was raised, completely blocked staining at 15 nM whereas the same concentration of two similar amidated carboxy termini (YPMRFamide, TRPRYamide) did not. Staining for each antiserum was unaffected by simultaneous incubation with the other two peptides. Sections were cut on a vibratome at 80 μm (c, d) or cut on a cryostat at 10 μm for subsequent autoradiography (b, d inset). In a and c, the morphological appearance at E54 of cells immunoreactive for either SRIF or NPY is often similar to that of the subplate cells shown in Fig. 1. Note the many beaded processes in the 80 μm sections, some of which can be followed for several millimeters both away from and up into the cortical plate. In b, some of the cells immunoreactive for SRIF are indeed subplate cells since they can be tagged with ^3H -thymidine given at E27 (thick arrows). Note that not all cells generated on E27 are SRIF-immunoreactive (curved arrow), nor are all SRIF-positive cells born at E27 (thin arrow). Subplate cells and marginal zone (future layer 1) cells have the same birthdates¹, and both populations can be immunoreactive for CCK. An example of a CCK-immunostained marginal zone cell at age P7 is shown in d. The inset shows a double-labelled marginal zone cells to show that the birthdate of some CCK-immunostained marginal zone cells is E27. Pial surface is always towards the top of the figure.



located in the white matter¹, it seemed likely that the peptide immunoreactive cells of the adult white matter are the residuum of the transient subplate cell population that have survived into adult life. When sections from developing brains between E50 and P7 were tested for neuropeptide immunoreactivity, specific immunostaining of cells in the telencephalon was found for neuropeptide Y (NPY), somatostatin (SRIF) and cholecystokinin (CCK). Figure 3 shows examples of cells in the subplate immunoreactive for SRIF (Fig. 3a) and NPY (Fig. 3c) at E54. Immunostaining for SRIF and NPY could first be detected by E50, but that for CCK could not be observed until around E60. Also, the majority of CCK-immunoreactive cells are found in the marginal zone (Fig. 3d and Fig. 4), whereas all of the NPY- and SRIF-immunoreactive cells are located in the subplate or deepest layers of the cortical plate.

Some if not all of these peptide-immunoreactive cells belong to the early generated population of subplate and marginal zone cells, for they can be tagged with ^3H -thymidine at E27. For example, the autoradiograph of Fig. 3b shows that some of the subplate cells with silver grains over their nuclei are also immunoreactive for SRIF at E54. In addition, double-labelled cells (Fig. 3b, large arrows) are intermixed with neurons born at the same time but not SRIF-immunoreactive (Fig. 3b, curved arrow), indicating that subplate neurons born at any particular time are heterogeneous with respect to transmitter phenotype. Figure 3b also shows that some of the SRIF immunoreactive cells are not labelled with ^3H -thymidine (thin arrows). Nevertheless, many of these immunostained but not thymidine-labelled cells are probably subplate neurons as only a small fraction of the entire subplate population can be labelled by a single ^3H -thymidine injection²³. Similar conclusions can be drawn for the NPY- and CCK-immunostained populations (Fig. 3d, inset).

To examine whether there is a relationship between peptide immunoreactivity and location within the telencephalon three

adjacent sections from a P7 cat brain were immunostained with one of the three antibodies against NPY, SRIF or CCK and the distribution of immunoreactive cells recorded. Figure 4 shows the results of this comparative analysis. Counts of the number of labelled cells per section indicate that about 75% of all NPY-immunoreactive cells are embedded within the white matter/lower subplate, whereas ~70% of all of the SRIF-immunoreactive cells are found more superficially in a region located immediately beneath the cortical plate: the upper subplate (see ref. 1 for definitions of the upper and lower subplate). Finally, 65% of all CCK-immunoreactive cells reside within the marginal zone, where they comprise the only peptide-immunoreactive population observed there. Thus cells immunoreactive for a given peptide tend to reside within a specific tangential domain of the telencephalon.

The results of this study show that the subplate cells, identified by ^3H -thymidine birthdating, are neurons. First, all are found to be immunoreactive for the neuron- and dendrite-specific marker MAP2. Second, these cells can project locally within the subplate and can extend long processes to the opposite hemisphere. Third, they resemble neurons ultrastructurally and receive synapses. Fourth, subpopulations are immunoreactive for at least one of three neuropeptides: CCK, SRIF and NPY.

The morphology of the early generated cells studied here is remarkably similar to that of Golgi-impregnated cells in previous studies of developing cortex. The marginal zone cells immunoreactive for CCK resemble the transient Cajal-Retzius cells^{14,24-27}. The morphology of many subplate neurons is similar to that of the Martinotti cell or interstitial neuron^{13,26,28,29}. We suggest that most if not all of these cell types belong to the early-generated, transient population that we have defined with the various double-label techniques of the present study. Although conclusive proof for this suggestion requires ^3H -thymidine identification of Golgi stained material, it is supported

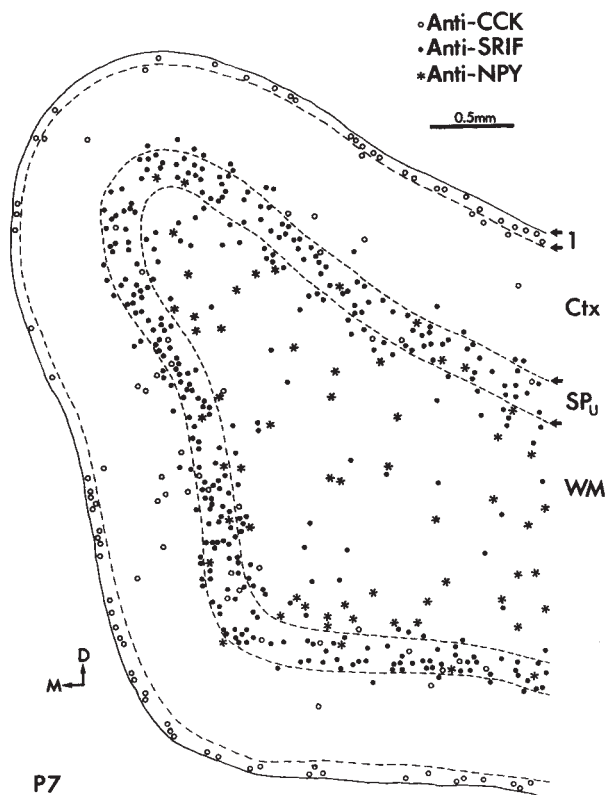


Fig. 4 The peptide immunoreactivity of subplate cells varies with their tangential position. Three adjacent 80- μ m-thick coronal sections through the visual cortex of a P7 cat were immunostained with one of the antisera against CCK, NPY or SRIF. Each section was drawn with a camera lucida attachment and the location within the telencephalon of all labelled cells with a given peptide immunoreactivity was marked. The three drawings were then superimposed. Symbols identify cells immunoreactive for a given peptide: open circles, CCK; closed circles, SRIF; asterisks, NPY. Note the exclusive presence of CCK immunostained cells in layer I, the clustering of SRIF immunostained cells in the upper subplate (SP_U), and the NPY immunostained cells deep in the white matter (WM). Ctx, cortex; M, medial; D, dorsal.

by two recent observations. Firstly, MAP2-immunoreactive cells thought to be Cajal-Retzius cells have been found in the marginal zone of mice embryos³⁰ and secondly in the telencephalon of human fetuses, some cells located in the subplate are also immunoreactive for SRIF³¹.

The subplate cells are neurons that receive synaptic inputs during development. The most likely sources of the presynaptic inputs are the ingrowing thalamocortical and corticocortical axons, which accumulate in the subplate when the subplate cells are present^{3-6,32,33}. Thus, the subplate cells may serve as the temporary targets of ingrowing afferent systems. If so, these cellular elements could represent part of an early and transient neural circuit that precedes the establishment of adult cortical connectivity. In addition, the distinct tangential domains occupied by cells of a given neuropeptide immunoreactivity suggest that different groups of subplate cells could serve distinct functions during the development of the mammalian cerebral cortex.

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1. Luskin, M. B. & Shatz, C. J. *J. Neurosci.* **5**, 1062-1075 (1985).
2. Sidman, R. L. & Rakic, P. *Brain Res.* **62**, 1-35 (1973).
3. Luskin, M. B. & Shatz, C. J. *Soc. Neurosci. Abs.* **10**, 1079 (1984).
4. Shatz, C. J. & Luskin, M. B. *J. Neurosci.* **6**, 3655-3668 (1986).
5. Rakic, P. *Phil. Trans. R. Soc. B278*, 245-260 (1977).
6. Lund, R. D. & Mustari, M. J. *J. comp. Neurol.* **173**, 289-306 (1977).
7. Molliver, M. E., Kostovic, I. & Van Der Loos, H. *Brain Res.* **50**, 403-407 (1973).
8. Kostovic, I. & Molliver, M. E. *Anat. Rec.* **178**, 395 (1974).
9. Cragg, B. G. *J. comp. Neurol.* **160**, 147-166 (1975).
10. Blue, M. E. & Parnavelas, J. G. *J. Neurocytol.* **12**, 599-616 (1983).
11. Blue, M. E. & Parnavelas, J. G. *J. Neurocytol.* **12**, 697-712 (1983).
12. Chun, J. J. M. & Shatz, C. J. *Soc. Neurosci. Abstr.* **9**, 692 (1983).
13. Kostovic, I. & Rakic, P. *J. Neurocytol.* **9**, 219-242 (1980).
14. Parnavelas, J. G. & Edmunds, S. M. *J. Neurocytol.* **12**, 863-871 (1983).
15. Luskin, M. B. & Shatz, C. J. *J. comp. Neurol.* **242**, 611-631 (1985).
16. De Camilli, P., Miller, P. E., Navone, F., Theurkauf, W. E. & Vallee, R. B. *Neuroscience* **11**, 819-846 (1984).
17. Bernhardt, R., Huber, G. & Matus, A. *J. Neurosci.* **5**, 977-991 (1985).
18. Laemle, L. K., Feldman, S. C. & Lichtenstein, E. *Brain Res.* **251**, 365-370 (1982).
19. Somogyi, P. *et al. J. Neurosci.* **4**, 2590-2603 (1984).
20. Hendry, S. H. C., Jones, E. G. & Emson, P. C. *J. Neurosci.* **4**, 2497-2517 (1984).
21. Hendry, S. H. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 6526-6530 (1984).
22. Chan-Palay, V., Allen, Y. S., Lang, W., Haesler, U. & Polak, J. M. *J. comp. Neurol.* **238**, 382-389 (1985).
23. Hickey, T. L., Whitehart, D. R., Jackson, C. A., Hitchcock, P. F. & Peduzzi J. D. *J. Neurosci. Methods* **8**, 139-147 (1983).
24. Raedler, E. & Raedler, A. *Anat. Embryol.* **154**, 267-284 (1978).
25. Caviness, V. S. Jr *Dev. Brain Res.* **4**, 293-302 (1982).
26. Ramon Y Cajal, S. *Histologie du System Nerveux de l'Homme et des Vertebres* Vol. 2 (Maloine, Paris, 1911).
27. Bradford, R., Parnavelas, J. G. & Lieberman, A. R. *J. comp. Neurol.* **176**, 121-132 (1977).
28. Marin-Padilla, M. Z. *Anat. EntwGes.* **134**, 125-142 (1971).
29. Marin-Padilla, M. Z. *Anat. EntwGes.* **136**, 125-142 (1972).
30. Crandall, J. E., Jacobson, M. & Kosik, K. S. *Dev. Brain Res.* **28**, 127-133 (1986).
31. Kostovic, I. & Fucic, A. *Soc. Neurosci. Abstr.* **11**, 352 (1985).
32. Wise, S. P. & Jones, E. G. *J. comp. Neurol.* **178**, 187-208 (1978).
33. Innocenti, G. M. *Science* **212**, 824-827 (1981).
34. McLean, I. W. & Nakane, P. K. *J. Histochem. Cytochem.* **22**, 1077-1083 (1974).
35. Adams, J. C. *J. Histochem. Cytochem.* **29**, 775 (1981).

A glucagon fragment is responsible for the inhibition of the liver Ca^{2+} pump by glucagon

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Glucagon specifically inhibits the Ca^{2+} pump in liver plasma membranes independently of adenylate cyclase activation¹. However, this inhibition is only observed at high concentrations of glucagon ($K_i = 0.7 \mu$ M). Moreover, in the presence of bacitracin, an inhibitor of glucagon degradation², the Ca^{2+} pump is no longer sensitive to glucagon³. These findings suggest that a fragment of glucagon might be the true effector of the liver Ca^{2+} pump. Pairs of basic amino acids are recognized as potential cleavage sites in post-translational processing of peptide hormones⁴⁻⁶. The glucagon molecule includes a dibasic doublet (Arg 17-Arg 18). Therefore, we have examined the action of glucagon(19-29) on the liver Ca^{2+} pump. This peptide was obtained from glucagon by tryptic cleavage and separated by reverse-phase high-performance liquid chromatography. We found that glucagon(19-29), which is totally ineffective in activating adenylate cyclase, inhibited both the Ca^{2+} -activated and Mg^{2+} -dependent ATPase activity ($(Ca^{2+}$ - Mg^{2+}) ATPase) and Ca^{2+} transport in liver plasma membranes with an efficiency 1,000-fold higher than that of glucagon. Glucagon(1-21) was completely inactive; glucagon(18-29) and glucagon(22-29) acted only as partial agonists of glucagon(19-29). These results indicate that glucagon(19-29), obtained by proteolytic cleavage of glucagon, is likely to be the active peptide involved in the inhibition of the liver Ca^{2+} pump. We suggest that glucagon may be a precursor of at least one biologically active peptide.

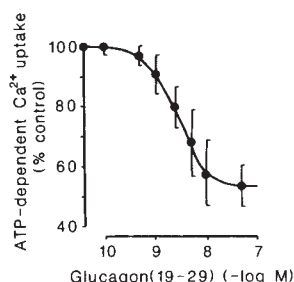


Fig. 1 Inhibition by glucagon(19-29) of the ATP-dependent Ca^{2+} uptake by liver plasma membrane vesicles. Results are the mean of five different experiments, done with five different plasma membrane vesicle preparations; bars, $\pm$ s.e.m.

Methods. Plasma membrane vesicles were prepared from liver of female albino Wistar rats (100–120 g body weight) according to Prpic *et al.*⁷, using a Percoll gradient. Glucagon(19-29) was prepared as described in Fig. 3 legend, from crystalline pancreatic glucagon provided by Novo Laboratories (Paris, Copenhagen). Ca^{2+} uptake was assayed in inside-out plasma membrane vesicles as previously described¹. Preincubation at 37 °C for 5 min of plasma membrane vesicles (120–320 μg protein) with glucagon(19-29) (0–50 nM) diluted in 50 mM Tris-HCl, pH 8, 0.01% bovine serum albumin (BSA) and 0.87 μM lactose, allowed the peptide to interact with intravesicular sites of inside-out vesicles because of the leakiness of vesicles. After 5 min, the incubation was run in 200 μl of assay medium containing 50 mM Tris HCl, pH 8, 0.01% BSA, 400 μM EGTA, 393 μM CaCl_2 (0.1 μM free Ca^{2+}), 2 $\mu\text{Ci ml}^{-1}$ of ^{45}Ca , 180 mM sucrose, 1 μM ruthenium red, 20 mM sodium azide, 4 mM Tris-oxalate, 10 mM MgCl_2 , with either no ATP added or 10 mM ATP. MgCl_2 in the assay medium is used to seal vesicles^{1,28}. After 15 min, 150 μl samples were filtered under vacuum on Millipore filters (HAWP 0.45 μM) that had been soaked in 0.25 M KCl for 1 h. The filters were washed three times with 5 ml iced 250 mM sucrose, 40 mM NaCl, dried, and counted in 10 ml of Beckman Ready Solv. The ATP-dependent Ca^{2+} uptake was determined from the difference in radioactivity bound to the filter in the presence and absence of ATP. Uptake is expressed as a percentage of control ATP-dependent Ca^{2+} uptake (6.1 ± 1.7 nmol Ca^{2+} per mg protein per 15 min).

The ATP-dependent Ca^{2+} uptake was studied in liver plasma membrane vesicles prepared according to Prpic *et al.*⁷. Addition of glucagon(19-29) (0–50 nM) to the assay medium resulted in a dose-dependent inhibition of the ATP-dependent Ca^{2+} uptake (Fig. 1). A maximal inhibition of 40–60% was attained with 10 nM glucagon(19-29), half-maximal inhibition occurring at 3 ± 0.3 nM glucagon(19-29).

From kinetic studies¹ and reconstitution experiments using the purified enzyme (in preparation), we have concluded that the high-affinity $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase is the enzyme responsible for Ca^{2+} transport. Accordingly, $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity was assayed in purified liver plasma membranes prepared according to Neville⁸ up to step 11. This preparation was chosen because it has been used for the characterization of numerous membrane enzymes and receptors^{9–13}. Glucagon(19-29) induced a dose-dependent inhibition of the $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity (Fig. 2). Maximal inhibition was 15–20% at 10 nM glucagon(19-29), and half-maximal inhibition occurred at 0.75 ± 0.25 nM glucagon(19-29). Inhibition was due to a decrease in maximal velocity of the reaction with no change in the apparent affinity of the enzyme for calcium (data not shown). Also, $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase was assayed in parallel with Ca^{2+} uptake in liver plasma membrane vesicles prepared according to Prpic *et al.*⁷. In these preparations, inhibition of $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase by glucagon(19-29) reached 15–20% at 10 nM glucagon(19-29), half-maximal inhibition being observed for 1 ± 0.25 nM glucagon(19-29) (data not shown).

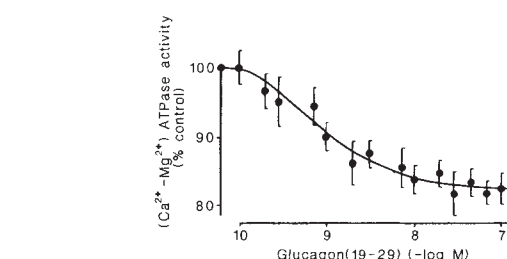


Fig. 2 Inhibition of $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity in purified liver plasma membranes by glucagon(19-29). Results are the mean of ten different experiments done with five different membrane preparations; bars, $\pm$ s.e.m.

Methods. Purified plasma membranes were prepared from liver of female albino Wistar rats (100–120 g body weight) according to Neville⁸ up to step 11. Plasma membranes (4–8 μg protein) were preincubated at 4 °C with glucagon(19-29) (0–0.1 μM) diluted in 50 mM Tris-HCl, pH 8, 0.01% BSA and 0.87 μM lactose. After 10 min, $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity was assayed as previously described¹. The assay medium contained in a final volume of 200 μl , 0.25 mM ATP, 50 mM Tris-HCl, pH 8, 400 μM EGTA and 393 μM CaCl_2 (0.1 μM free Ca^{2+}). After 10 min incubation at 30 °C, aliquots were assayed for P_i by colorimetric determination²⁹. Basal activity was estimated for each point by omitting CaCl_2 from the assay medium and $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity, calculated by subtracting values obtained in the presence of chelator alone from those obtained with chelator plus CaCl_2 , is expressed as a percentage of control $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity (1.2 ± 0.2 nmol P_i per mg protein per 10 min).

Native glucagon caused 25 and 50% maximal inhibition of liver $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase and Ca^{2+} transport respectively, but its apparent affinity for the Ca^{2+} pump is in the micromolar range (ref. 1, Table 1). Therefore, it appears that glucagon(19-29) is 1,000-fold more efficient than native glucagon in inhibiting the liver Ca^{2+} pump. To assess the specificity of the action of glucagon(19-29), we investigated the action of three other glucagon fragments: glucagon(18-29), glucagon(22-29) and glucagon(1-21). Glucagon(18-29) was tenfold less potent than glucagon(19-29) in inhibiting $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase (Table 1 and Fig. 3). Glucagon(22-29) produced only a 5–15% maximal inhibition of $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase, with a low potency (1 μM), similar to that of native glucagon (Table 1). The amino-terminal (1-21) fragment of glucagon had no effect on $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase (Table 1) nor did it antagonize inhibition induced by either native glucagon or glucagon(19-29).

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Table 1 Effects of various glucagon fragments on $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity in liver plasma membranes

	Glucagon(19-29)	Glucagon(18-29)	Glucagon	Glucagon(22-29)	Glucagon(1-21)
K_i (nM)	0.75 ± 0.5	7.5 ± 2.5	700 ± 300	$1,000 \pm 300$	10,000
Inhibition (% maximal)	20 ± 5	15 ± 5	25 ± 10	10 ± 5	—

The $(\text{Ca}^{2+}\text{-Mg}^{2+})$ ATPase activity was measured as in Fig. 2 legend, in the presence of 0–10 μM of either glucagon(19-29), glucagon(18-29), glucagon, glucagon(22-29) or glucagon(1-21). A 10-min preincubation at 4 °C of plasma membranes (3–8 μg protein) with the peptide was used, followed by a 10-min incubation at 30 °C, under the conditions described in the legend to Fig. 2. Results are the mean of four different experiments using three different plasma membrane preparations, and are expressed as a percentage of control activity (1.3 ± 0.2 $\mu\text{mol Pi}$ per mg protein per 10 min). Sources: glucagon(1-21), a gift from Novo Laboratories; glucagon(22-29) from Peninsula Laboratories; glucagon(19-29) and glucagon(18-29), prepared from glucagon as described in Fig. 3 legend.

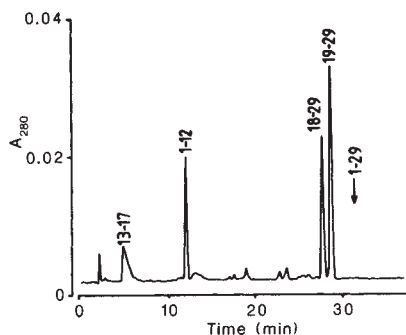


Fig. 3 Preparation of glucagon(19-29) and glucagon(18-29) by HPLC. Arrow, elution volume of intact glucagon(1-29).

Methods. Crystalline porcine pancreatic glucagon was digested for 1 h at 37 °C in 50 mM Tris HCl, 1 mM CaCl₂, pH 7.5, with tosyl-phenylethyl-chloromethyl-ketone (TPCK)-treated trypsin (Sigma) at a 1:100 (w/w) enzyme/substrate ratio. After boiling for 3 min, the peptide mixture was separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Waters apparatus equipped with a μ Bondapak C₁₈ column run at 1.5 ml min⁻¹ in 0.1% trifluoroacetic acid. The peptides were eluted with a linear gradient (0–50% in 50 min) of acetonitrile containing 0.05% ethyl-acetate³⁰. Peptides were monitored by UV absorption at 214 and 280 nm, UV spectra were obtained on-line from the HPLC peaks using a Hewlett-Packard model 8450A diode array spectrophotometer. The purity of the peptide fragments was assessed by comparing their UV spectra (not shown) with theoretical spectra obtained with a computer databank (D.B. and M.D., manuscript in preparation) and by amino-acid analysis performed with RP-HPLC of OPA derivatives³¹. The results obtained (theoretical values in parentheses) were: glucagon(19-29): Ala 1.1 (1), Asp 2.2 (2), Glu 2.4 (2), Phe 1.3 (1), Leu 1.2 (1), Met 0.8 (1), Arg 0.14 (0), Thr 1.1 (1), Val 0.9 (1); Glucagon(18-29): Ala 1.3 (1), Asp 2 (2), Glu 2.2 (2), Phe 1.1 (1), Leu 0.9 (1), Met 0.8 (1), Arg 1.3 (1), Thr 1.3 (1), Val 0.9 (1). The presence of intact tryptophan in both peptides was assessed by their UV spectra (not shown).

The integral glucagon molecule is required for full activation of adenylate cyclase. In particular, neither glucagon(1-21)¹⁴ nor glucagon(22-29)¹⁵ can stimulate adenylate cyclase activity. We did not observe any effect of glucagon(19-29) (0–0.1 mM) on adenylate cyclase activity (not shown). This is in keeping with our previous observation that inhibition of the Ca²⁺ pump by glucagon is independent of cyclic AMP production¹.

Our results clearly indicate that nanomolar concentrations of glucagon(19-29) specifically inhibit the Ca²⁺ pump in liver plasma membranes. Furthermore, the present data show that the amino-terminal residues in glucagon(19-29) are essential. Indeed, the addition of an arginine residue (peptide (18-29)) or the suppression of residues 19–21 (peptide (22-29)) resulted in a dramatic loss of potency. Thus, glucagon(19-29) appears to be the molecular form of glucagon responsible for the inhibition of the calcium pump in the liver plasma membrane. The metabolic consequences of the action of glucagon(19-29) on liver cells, in particular its effect on the glycogenolytic pathway, remain to be elucidated, as does the nature of the specific receptor which recognizes this peptide.

Two hypotheses can be raised concerning the physiological precursor of glucagon(19-29). Firstly, glucagon(19-29) could be released during the processing of proglucagon as are other glucagon-related peptides^{16–20}. Alternatively, glucagon(19-29) could be generated locally upon interaction of the circulating glucagon with hepatocytes. The existence of a trypsin-like protease associated with liver plasma membranes has been demonstrated²¹. Conversion of peptide hormones by the target tissue has already been described for growth hormone²², proinsulin²³, angiotensin¹²⁴, and arginine vasopressin²⁵. Also, cleavage of growth hormone²⁶ and arginine vasopressin²⁵ has been shown to generate new biological activities.

It was generally accepted that adenylate cyclase activation was the only enzymatic pathway regulated by glucagon. Wakelam *et al.*²⁷ recently reported that subnanomolar concentrations of glucagon cause a breakdown of inositol phospholipids independently of cyclic AMP production suggesting that glucagon may also regulate phospholipase C. Our findings provide a new mechanism by which glucagon may regulate liver cell metabolism through an enzymatic pathway distinct from adenylate cyclase or phospholipase C, and point out the possible role of glucagon as a precursor of peptides with new biological activities.

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1. Lotersztajn, S., Eppard, R. M., Mallat, A. & Pecker, F. *J. biol. Chem.* **259**, 8195–8201 (1984).
2. Desbuquois, B., Krug, F. & Cuatrecasas, P. *Biochim. biophys. Acta* **343**, 101–120 (1974).
3. Mallat, A., Pavoin, C., Lotersztajn, S. & Pecker, F. *Fedn Proc.* **44**, 1392 (1985).
4. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J. & Tager, H. S. *Ann. N.Y. Acad. Sci.* **343**, 1–16 (1980).
5. Patzelt, C. & Schiltz, E. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5007–5011 (1984).
6. Georges, S. K., Utenthal, L. O., Ghiglione, M., Bloom, S. R. *FEBS Lett.* **192**, 275–278 (1985).
7. Pripic, V., Green, K. C., Blackmore, P. F. & Exton, J. H. *J. biol. Chem.* **259**, 1382–1385 (1984).
8. Neville, D. M. *Biochim. biophys. Acta* **154**, 540–552 (1968).
9. Hanoune, J., Lacombe, M.-L. & Pecker, F. *J. biol. Chem.* **250**, 4569–4574 (1978).
10. Guellaen, G., Yates-Aggerbeck, M., Vauquelin, G., Strosberg, D. & Hanoune, J. *J. biol. Chem.* **253**, 1114–1120 (1978).
11. Clarke, W. R., Jones, L. R. & Lefkowitz, R. J. *J. biol. Chem.* **253**, 5976–5979 (1978).
12. El-Refaei, M. F., Blackmore, P. F. & Exton, J. H. *J. biol. Chem.* **254**, 4375–4386 (1979).
13. Billon, M. C., Dupre, G., Hanoune, J. *Molec. cell. Endocr.* **18**, 99–108 (1980).
14. Frandsen, E. K., Thim, L., Moody, A. J. & Markussen, J. *J. biol. Chem.* **260**, 7581–7584 (1985).
15. Wright, D. E., Hruby, V. J. & Rodbell, M. *J. biol. Chem.* **253**, 6338–6340 (1978).
16. Heinrich, G., Gros, P., Habener, J. F. *J. biol. Chem.* **259**, 14082–14087 (1984).
17. Bell, G. I., Santerre, R. F. & Mullenbach, G. T. *Nature* **302**, 716–718 (1983).
18. Jarrousse, C. *et al. FEBS Lett.* **188**, 81–84 (1985).
19. Bataille, D. *et al. FEBS Lett.* **146**, 79–86 (1982).
20. Martinez, J. & Potier, J. *Trends pharmac. Sci.* **4**, 139–147 (1986).
21. Tanaka, K., Nakamura, T. & Ichihara, A. *J. biol. Chem.* **261**, 2610–2615 (1986).
22. Schepper, J. M., Hughes, E. F., Postel-Vinay, M. C. & Hughes, J. P. *J. biol. Chem.* **259**, 12945–12948 (1984).
23. Peavy, D. E., Brunner, M. R., Duckworth, W. C., Hooker, C. S. & Frank, B. H. *J. biol. Chem.* **260**, 13989–13994 (1985).
24. Snyder, R. A., Watt, K. W. K. & Wintroub, B. U. *J. biol. Chem.* **260**, 7857–7860 (1985).
25. Burbach, J. P. H., Kovacs, G. L., De Wied, D., Van Nispen, J. W. & Greven, H. M. *Science* **221**, 1310–1312 (1983).
26. Maciag, T. *et al. J. biol. Chem.* **255**, 6064–6070 (1980).
27. Wakelam, J. O., Murphy, G. J., Hruby, V. J. & Houslay, M. D. *Nature* **323**, 68–71 (1986).
28. Cockroft, S. & Gomperts, B. D. *Nature* **314**, 534–536 (1985).
29. Kallner, A. *Clin. chim. Acta* **59**, 35–39 (1975).
30. Bataille, D., Coudray, A. M., Carlqvist, M., Rosselin, G. & Mutt, V. *FEBS Lett.* **146**, 73–78 (1982).
31. Fleury, M. O. & Ashley, D. V. *Analyt. Biochem.* **133**, 330–335 (1983).

Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence

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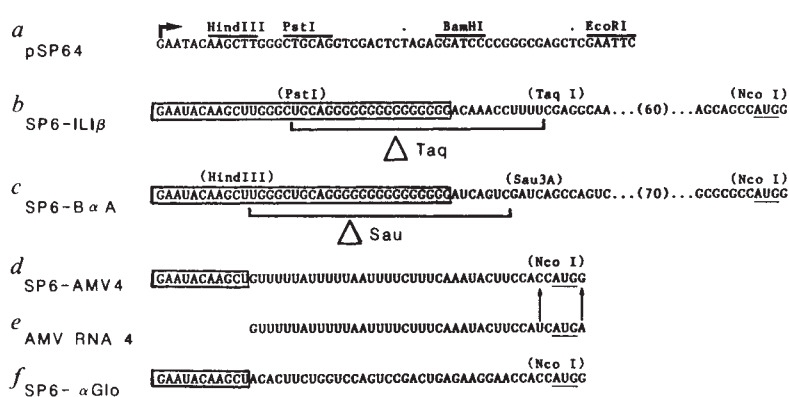
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Eukaryotic messenger RNAs are translated with unequal efficiencies *in vivo*^{1,2} and *in vitro*^{3–5} and the molecular basis of this phenomenon is not understood. As an approach to understanding the role of the 5' untranslated leader sequence in regulating mRNA translational efficiency, chimaeric mRNAs have been generated by joining a heterologous leader to complementary DNA (cDNA) sequences, followed by *in vitro* transcription using SP6 RNA polymerase and *in vitro* protein synthesis. We used the untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV

Fig. 1 Nucleotide sequences of the 5' untranslated leaders. *a*, As a reference, the transcriptional start site (arrow) and the polycloning region of plasmid SP64 are shown; *b*, *c*, corresponding regions of SP6-IL1 β and SP6-B α A. Square brackets, nucleotides deleted during construction of Δ Taq-IL1 β and Δ Sau-B α A; numbers in parentheses, nucleotides in the untranslated leader of IL-1 β or B α A which are present in the construct but not shown in the figure. *d*, Corresponding regions of chimaeric AMV mRNAs; and *e* of native AMV RNA 4 strain 425²⁶. Upward arrows, nucleotides changed during construction of the SP6-AMV4 untranslated leader (*d*). *f*, Corresponding regions of SP6-rabbit α -globin. In all the constructs, underlines indicate the initiation codon for protein synthesis and boxed nucleotides indicate vector sequences upstream of the 5' end of the cDNA inserts which are transcribed by SP6 polymerase. Identical nucleotides in each sequence have been aligned vertically. To avoid ambiguities in the data which might be attributed to codon context surrounding the initiator AUG^{11,27,28}, the leader-coding sequence junction of all DNA constructs has been standardized upon the recognition sequence for the restriction enzyme *Nco*I (CCATGG, where ATG is the initiation codon). Barley α -amylase⁸, human interleukin 1 β ⁹ and α -globin²⁹ cDNAs have naturally occurring *Nco*I sites separating the 5' untranslated leader sequence and the protein coding region (*b*, *c*, *f*).

Methods. SP6-IL1 β and SP6-B α A were constructed by inserting the human interleukin 1 β cDNA⁹ and the type A barley α -amylase cDNA⁸ into the *Pst*I-*Bam*HI or *Pst*I sites, respectively, of pSP64 (Promega Biotec). To construct Δ Taq-IL1 β , a 30-base-pair *Pst*I-*Taq*I fragment was removed³⁰ from SP6-IL1 β . A 31-base-pair *Hind*III-*Sau* 3A fragment was removed from SP6-B α A to create Δ Sau-B α A. An oligodeoxyribonucleotide representing the sequence of the AMV RNA 4 leader (modified to create an *Nco*I site at the 3' terminus) was synthesized as complementary strands which were annealed and ligated into the *Hind*III-*Nco*I sites of SP6-IL1 β or SP6-B α A to create AMV-IL1 β or AMV-B α A. An oligodeoxyribonucleotide representing the rabbit α -globin untranslated leader²⁹ was similarly synthesized and inserted into SP6-B α A to create α Glo-B α A.



RNA 4), a well-translated, highly competitive message^{5,6}, to replace the leader sequence of barley α -amylase (B α A) and human interleukin 1 β (IL-1 β) cDNAs. Deletion of transcribed vector sequences and replacement of the native untranslated region with the AMV RNA 4 leader can result in as much as a 35-fold increase in mRNA translational efficiency; moreover, the translational efficiency of the chimaeric mRNAs containing the AMV RNA 4 leader is at least as great as that of virion RNA 4. The results suggest that the chimaeric AMV-mRNAs have either a higher relative affinity or a diminished requirement for a limiting component(s) of the translational machinery; in addition, it may be feasible, through use of heterologous leader sequences, to increase expression of engineered genes or cDNAs without changing the antigenic or biological properties of the encoded protein.

Because of the correlation between absence of stable 5' secondary structure and enhanced translational efficiency of AMV RNA 4⁷, we decided to test the role of the AMV RNA 4 untranslated leader in facilitating translation by transferring the sequence to the 5' terminus of other mRNAs. The SP6-IL1 β and SP6-B α A subclones contain a 5' G₁₅ sequence added during cloning^{8,9}; therefore, the SP6 RNA transcripts contain 20 nucleotides of 5' SP6 vector sequence followed by 15 guanosine residues preceding the cDNA sequence (Fig. 1*b*, *c*). The 5' poly(G) sequence was deleted from the IL-1 β cDNA by excising a 30-base-pair fragment ending in a *Taq*I site just inside the untranslated leader (Fig. 1*b*). The Δ Taq-IL1 β mRNA was translated with greater efficiency in both protein-synthesizing systems than the SP6-IL1 β mRNA containing the poly(G) sequence (Fig. 2*a*, *b*). In a third construct, the AMV RNA 4 untranslated leader was substituted for the IL-1 β leader by first excising a *Hind*III-*Nco*I fragment from SP6-IL1 β DNA, and then inserting a 37-base-pair AMV RNA 4 leader oligonucleotide (Fig. 1*d*). The 5' nucleotide sequence of the chimaeric SP6 mRNAs containing the AMV RNA 4 leader is shown in Fig. 1*d*. The translational efficiency of the chimaeric AMV-IL1 β mRNA increased 2–2.5-fold over the Δ Taq-IL1 β mRNA and the total increase in translational efficiency resulting from removal of the homopolymer tail and native IL-1 β leader, followed by substitution of the AMV RNA 4 untranslated leader sequence, was ~6–7-fold (Fig. 2*a*, *b*).

Qualitatively similar results were obtained using the B α A cDNA, although the SP6-B α A mRNA stimulated protein syn-

thesis *in vitro* minimally, and deletion of the 5' poly(G) sequence had a more dramatic effect on translational efficiency than observed with the IL-1 β mRNA (Fig. 2*c*, *d*). The G₁₅ sequence was removed from the SP6-B α A subclone by excising a 31-base-pair fragment ending at a *Sau*3A site immediately within the untranslated leader sequence (Fig. 1*c*), and as shown in Fig. 2*c*, *d*, the corresponding Δ Sau-B α A mRNA translated well in both reticulocyte lysate and wheat-germ extract. Substitution of the AMV RNA 4 leader for the B α A leader was performed as for the IL-1 β construct. The chimaeric AMV-B α A mRNA was translated with 2–3-fold greater efficiency than Δ Sau-B α A, giving a total increase, in comparison with SP6-B α A, of ~10-fold in the wheat-germ extract and 35-fold in the reticulocyte lysate (Fig. 2*c*, *d*). Data from control experiments demonstrated that all mRNAs were stable during the translation experiments and that unequal capping efficiencies did not form the basis of the differential translational efficiencies. Analysis of the translation products by SDS-polyacrylamide gel electrophoresis verified that the qualitative patterns of proteins translated from the chimaeric or non-chimaeric mRNA were similar and that the composition of the untranslated leader sequence had a notable effect upon the quantity of protein synthesized (data not shown).

A detailed analysis of codon context requirements for optimal translation of an mRNA in transfected COS cells^{10,11} found that rat preproinsulin mRNA containing an adenosine (A) residue at position -3 relative to the initiation codon was translated with greater efficiency than the same mRNA with a guanosine (G) at position -3. To test the possibility that the increased translational efficiency of chimaeric mRNAs was due only to changing the G at -3 present in IL-1 β and B α A to the A found in AMV RNA 4, an oligodeoxyribonucleotide representing the untranslated leader of rabbit α -globin (Fig. 1*f*) was used to replace the B α A leader. The α -globin leader is characterized by an A at position -3 and the nucleotide sequence is the same length as the AMV RNA 4 leader (Fig. 1*d*, *f*); moreover, the α -globin untranslated leader ends at an *Nco*I restriction site which is located within the optimal codon consensus sequence and putative ribosome binding site^{10,11} (Fig. 1*f*). If the preferred codon context found in the chimaeric AMV-mRNAs by itself resulted in increased translational efficiency, then use of the rabbit α -globin leader sequence, with its ideal context, would

Fig. 2 Analysis of the translational efficiency of SP6 mRNAs and chimaeric AMV-mRNAs *in vitro*. Incorporation of ^{35}S -methionine is shown as a function of RNA concentration for IL-1 β or B α A mRNAs translated in either the rabbit reticulocyte lysate (a, c) or wheat-germ extract (b, d).

Methods. Capped SP6 mRNAs were transcribed from linearized DNAs³¹, and low-molecular-weight materials were removed by passing the DNase-treated transcription reaction through two successive Sephadex G-50 spun columns³⁰. The eluate was then extracted twice with phenol/chloroform, once with ether, and nucleic acids were precipitated with ethanol from 2.5 M ammonium acetate. Translations were performed essentially according to the manufacturer's recommendations. Wheat-germ translations were prepared using 5 μl of nuclease-treated wheat-germ extract (Amersham) in a total volume of 10 μl containing 105 mM potassium acetate, 2 mCi ml⁻¹ ^{35}S -methionine, and the reaction mixture was incubated for 30 min at 25 °C. Reticulocyte translations were performed using 7 μl nuclease-treated rabbit reticulocyte lysate (Promega Biotec) in a total reaction volume of 11 μl containing 2 mCi ml⁻¹ ^{35}S -methionine, and the reactions were incubated for 15 min at 30 °C. In all examples, the salt conditions were optimized for the RNAs being translated, and incorporation of isotope was linear with respect to time during the incubation period. Duplicate 1- μl aliquots were removed for quantitation of hot-acid-precipitable radioactivity. The counts per min in the water control translations (without added mRNA) have been subtracted before plotting (reticulocyte lysate: 7,220 c.p.m. μl^{-1} ; wheat-germ extract: 2,580 c.p.m. μl^{-1}).

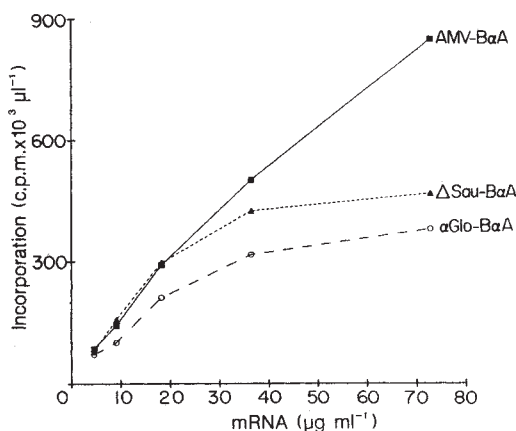
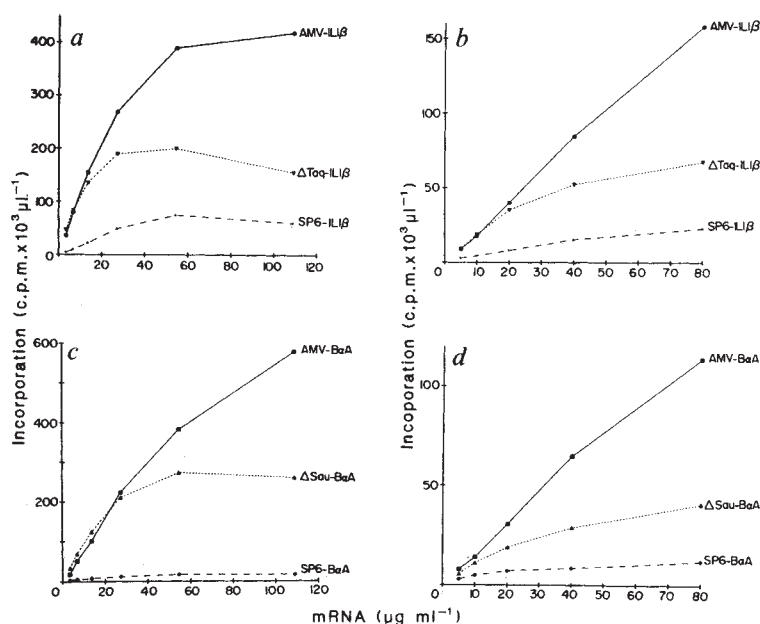


Fig. 3 The enhanced translational efficiency of the chimaeric AMV-mRNAs is a result of a feature of the leader other than the purine at position -3 and codon context effects.

Methods. Barley α -amylase mRNAs containing either the AMV-, ΔSau -, or α -Glo-untranslated leaders (see Fig. 1 for details) were translated in the reticulocyte lysate as in Fig. 2. Both the AMV- and α -globin untranslated leaders have perfect consensus sequences surrounding the initiation codon^{10,11,27} whereas the ΔSau leader has a guanosine residue at position -3. (Control without mRNA: 14,500 c.p.m.)

also be expected to increase translation of chimaeric mRNAs. However, the translational efficiency of the chimaeric $\alpha\text{Glo-B}\alpha\text{A}$ was significantly less than that of AMV-B αA and similar to $\Delta\text{Sau-B}\alpha\text{A}$ (Fig. 3), showing that the increased translational efficiency of the chimaeric AMV-RNAs (Fig. 2) is related to features of the leader other than the purine at position -3 and codon context effects.

When translated at concentrations $\geq 20 \mu\text{g ml}^{-1}$, the chimaeric AMV-mRNAs stimulate further incorporation of ^{35}S -methionine, whereas protein synthesis directed by the non-chimaeric mRNA reaches a plateau. These results suggest that a component required for efficient translation of the non-chimaeric mRNAs becomes limiting at high mRNA concentrations. In contrast to poliovirus infection¹², plant RNA viruses

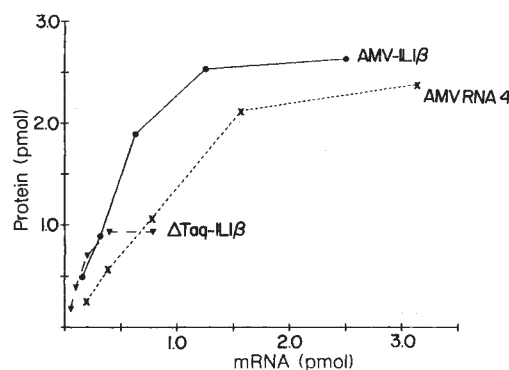


Fig. 4 The translational efficiency of the chimaeric AMV-mRNAs is similar to that of virion AMV RNA 4. Translation of AMV-IL1 β and $\Delta\text{Taq-IL1}\beta$ mRNAs in the reticulocyte lysate was compared to that of an SP6 transcript of the AMV RNA 4 cDNA. This SP6-AMV 4 mRNA translates with efficiency equal to that of virion RNA 4¹⁷. To normalize the data for differences in the length of the mRNAs and for the methionine content of the proteins, quantities have been expressed in moles.

Methods. Plasmid pSP65A4, yielding full-length transcripts of AMV RNA4¹⁷ was linearized with *Sma*I, and capped messenger RNAs were transcribed and translated as in Fig. 2.

do not cause a 'shut-off' of host protein synthesis¹³, however, coat protein mRNAs of many viruses possess properties providing for enhanced translation¹⁴⁻¹⁶, and coat protein is synthesized in massive quantities. Loesch-Fries *et al.*¹⁷ have reported that mRNA transcribed from plasmid pSP654A4, containing the complete AMV RNA 4 cDNA, translates with the same efficiency as virion mRNA. The translational efficiencies of AMV-IL1 β , $\Delta\text{Taq-IL1}\beta$, and AMV RNA 4 (from plasmid pSP65A4) were compared in the reticulocyte system (Fig. 4). The translational efficiency of the chimaeric AMV-IL1 β was slightly greater than that of AMV RNA 4, but that of $\Delta\text{Taq-IL1}\beta$, although similar to AMV-IL1 β at low mRNA concentrations, failed to increase stimulation of protein synthesis at high concentrations. The translational properties of the chimaeric AMV-

mRNAs seem to mimic those of the viral coat protein mRNAs through a characteristic elevated mRNA saturation concentration and highly efficient, competitive protein synthesis^{5,6,18}.

These studies support several conclusions regarding the role of the 5' untranslated leader sequence. First, the untranslated leader contains information which regulates both translational efficiency and the mRNA saturation point during translation *in vitro*. Second, the presence of the poly(G) region in the untranslated leader inhibits translation of the mRNA in a manner suggested by other work¹⁹. Comparison of the translational efficiencies of SP6-IL1 β and SP6-B α A (and L.G. and S.A.J. unpublished data) suggest that the poly(G) region is not inherently inhibitory, but may inhibit translation through interactions with other sequences within the mRNA. Third, an adenosine residue at position -3 relative to the AUG initiation codon is not necessarily correlated with optimal translational efficiency^{3,4,11} (Fig. 3). Regulatory regions in addition to codon context are therefore important in determining mRNA translational efficiency. Fourth, the AMV RNA 4 untranslated leader sequence affects the translational characteristics of the mRNA even when located 11 nucleotides downstream of the 5' cap structure (Fig. 1). This result suggests that the enhanced translational efficiency of the chimaeric AMV-mRNAs is not signalled by a unique nucleotide sequence located directly at the 5' mRNA terminus, but may relate to the secondary structure of the untranslated leader and the potential for formation of stable base pairs within the leader and between the leader and the 5' proximal coding sequence^{20,21}.

Messenger RNA secondary structure has been suggested repeatedly as an important factor in determining mRNA translational efficiency^{6,20-22}; however, little is known about how cells discriminate among mRNAs for translation. AMV RNA 4, which is principally unstructured at the 5' terminus⁷ may not require the 'unwinding' activity of the cap-binding protein complex^{21,23}; moreover, the mRNA is indeed translated in extracts prepared from poliovirus-infected HeLa cells where the high-molecular-weight component of the cap-binding complex is inactivated by proteolytic cleavage²⁴. Secondary structure analyses indicate that the single-stranded nature of the AMV RNA 4 leader is maintained in the chimaeric AMV-mRNA constructs²⁵. Our data are consistent with the hypothesis^{7,21} that mRNAs containing the AMV RNA 4 untranslated leader have a diminished requirement for a factor such as cap-binding protein complex, and that this diminished requirement is at least partially responsible for efficient, competitive translation and maximal synthesis at high mRNA concentration. The data also indicate that regulatory information carried in RNA may be transferred to heterologous sequences; this approach may also be useful for optimizing translational efficiency of transfected sequences *in vivo*, where the non-coding nature of the leader sequence may be suitable for increasing expression without altering the biological or antigenic properties of the translated protein.

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- Herson, D., Schmidt, A., Seal, S. N., Marcus, A. & van Vloten Doting, L. *J. biol. Chem.* **254**, 8245-8249 (1979).
- Godefroy-Colburn, T., Thivent, C. & Pinck, L. *Eur. J. Biochem.* **147**, 541-548 (1985).
- Gehrke, L., Auron, P. E., Quigley, G. Q., Rich, A. & Sonenberg, N. *Biochemistry* **22**, 5157-5164 (1983).
- Rogers, J. C. & Milliman, C. *J. biol. Chem.* **258**, 8169-8174 (1983).
- Auron, P. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 7907-7911 (1984).
- Kozak, M. *Nature* **308**, 241-246 (1984).
- Kozak, M. *Cell* **44**, 283-292 (1986).
- Ehrenfeld, E. *Cell* **28**, 435-436 (1982).
- Van Vloten-Doting, L. & Neeleman, L. in *Translation of Plant Virus RNAs* (eds Parthier, B. & Boulter, D.) 337-367 (Springer, New York, 1982).
- Zagorski, W. *Eur. J. Biochem.* **86**, 465-472 (1978).
- Joshi, S. & Haenni, A.-L. *FEBS Lett.* **177**, 163-174 (1984).
- Atabekov, J. G. & Morozov, S. Yu. *Adv. Virus Res.* **25**, 1-91 (1979).
- Loesch-Fries, L. S., Jarvis, N. P., Krahn, K. J., Nelson, S. E. & Hall, T. C. *Virology* **146**, 177-187 (1985).
- Friesen, P. D. & Rueckert, R. R. *J. Virol.* **49**, 116-124 (1984).
- Gough, N. M., Metcalf, D., Gough, J., Grail, D. & Dunn, A. R. *EMBO J.* **4**, 645-653 (1985).
- Iserentant, D. & Fiers, W. *Gene* **9**, 1-12 (1980).
- Sonenberg, N., Guertin, D., Cleveland, D. & Trachsel, H. *Cell* **27**, 563-572 (1981).
- McGarry, T. J. & Lindquist, S. *Cell* **42**, 903-911 (1985).
- Ray, B. K. *et al. J. biol. Chem.* **260**, 7651-7658 (1985).
- Sonenberg, N., Guertin, D. & Lee, K. A. W. *Molec. cell Biol.* **2**, 1633-1638 (1982).
- Gehrke, L. *Gene Analysis Techniques* **3**, 45-52 (1986).
- Brederode, R. T., Koper-Zwarthoff, E. C. & Bol, J. F. *Nucleic Acids Res.* **8**, 2213-2223 (1980).
- Kozak, M. *Microbiol. Rev.* **47**, 1-45 (1983).
- Kozak, M. *Nature* **308**, 241-246 (1984).
- Lockard, R. E. & RajBhandary, U. L. *Cell* **9**, 747-760 (1976).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning; a Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
- Edery, I. & Sonenberg, N. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7590-7594 (1985).

Inhibition of alloreactive cytotoxic T lymphocytes by peptides from the α_2 domain of HLA-A2

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Class I major histocompatibility complex (MHC) molecules function in the recognition of antigens by cytotoxic T lymphocytes (CTL)^{1,2}. Although this biological role is firmly established and much has been learnt about their structure and polymorphic variation³⁻⁶, little is known of the regions of class I molecules that are involved in functional interactions with components of the T-cell surface. Here we show that peptides derived from residues 98-113 of the α_2 domain of HLA-A2 specifically inhibit the recognition of target cells by many HLA-A2-specific CTL. In addition to identifying a region that is probably involved in binding the T-cell receptor these results raise the possibility that alloreactive CTL may recognize degraded fragments of class I histocompatibility antigens.

Class I MHC molecules control the recognition of membrane-associated antigens by CTL. For example virus-specific CTL recognize a particular combination of a viral antigen and a class I molecule^{1,7}. This phenomenon of MHC restriction is demonstrable because of the large polymorphism of class I molecules in most mammalian species. As a consequence class I molecules are major transplantation antigens and the targets for alloreactive CTL^{8,9}. Recent experiments have shown that the antigen receptors of virus-specific CTL preferentially recognize degraded fragments of viral proteins that become membrane associated^{7,10}. Whether alloreactive CTL recognize intact and/or degraded class I molecules is not known.

CTL target specificity is sensitive to changes in the structure of human class I molecules (HLA-A,B,C) and can discriminate between serologically identical molecules^{2,11-13}. Structural comparisons of such molecules have identified residues where substitutions affect the recognition by specific CTL. Analysis of H-2K^b

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- Cordell, B. *et al. Cell* **31**, 531-542 (1982).
- Alton, T. H. & Lodish, H. F. *Cell* **12**, 301-310 (1977).
- Lodish, H. F. *A. Rev. Biochem.* **45**, 39-72 (1976).
- Kabat, D. & Chappel, M. R. *J. biol. Chem.* **252**, 2684-2690 (1977).

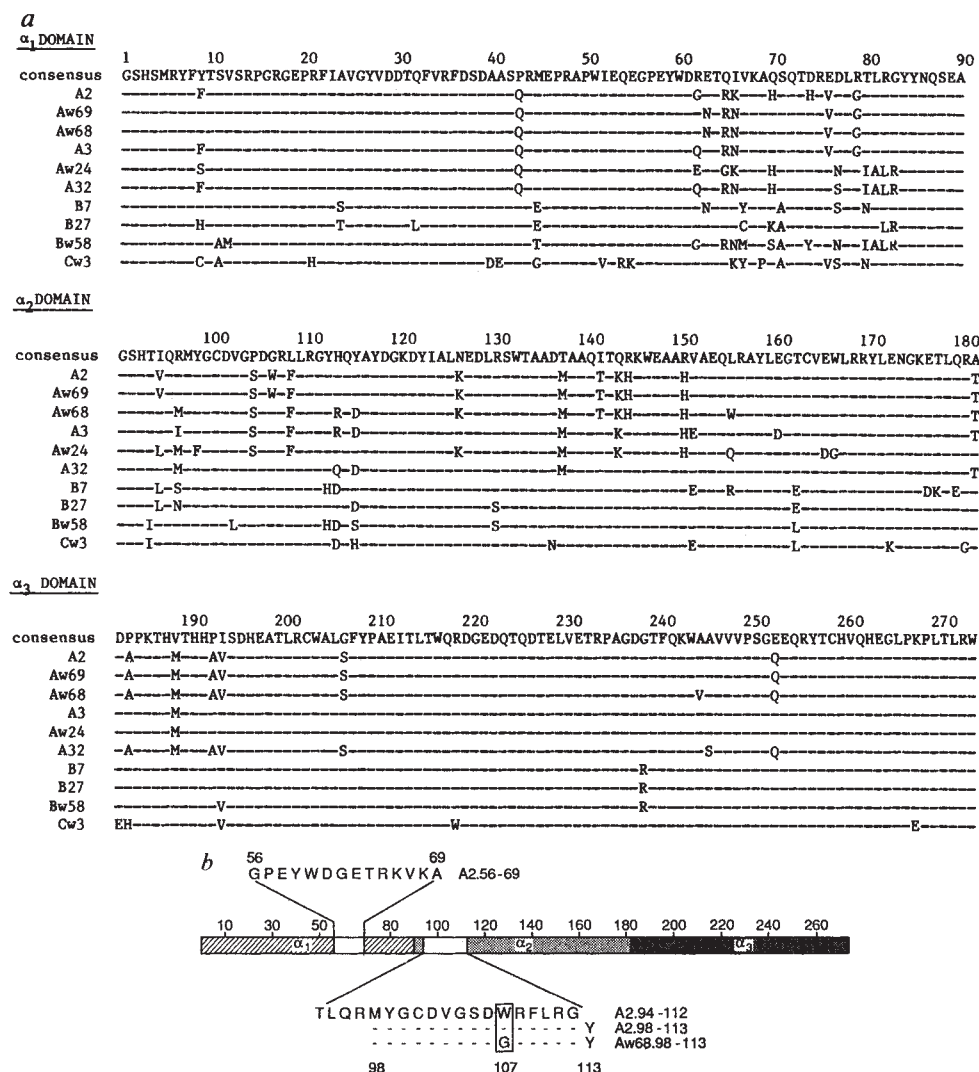


Fig. 1 *a*, The amino-acid sequences of the three extracellular domains (α_1 , α_2 , α_3) of HLA-A2, Aw69 and Aw68 are compared to seven other HLA-A,B,C molecules. The heavy chain which associates with β_2 -microglobulin consists of three amino-terminal extracellular domains (α_1 , α_2 , α_3) a transmembrane anchoring region and a short cytoplasmic domain. The three extracellular domains are thought to interact with CTL and only they are depicted. Sequence variability is concentrated in the α_1 and α_2 domains. With the exception of A32³⁹, Aw68 and Aw69²³, the sequences were obtained from a compilation in ref. 22 which also contains the primary references for each sequence. The consensus sequence is derived from a total of 23 HLA-A,B,C sequences by taking the most frequent residue at each position. Sequences are given in the standard one-letter code and identity with the consensus is indicated by a dash. *b*, Diagram showing the regions of the HLA-A,B,C heavy chain from which the peptides used in this study were derived. Peptides were synthesized by standard solid-phase methods³⁸ and their sequences are given in the standard one-letter code. The sequences of A2.98-113 and Aw68.98-113 were confirmed by automatic Edman degradation using a gas phase sequencer and shown to be >95% pure.

Table 1 Specificity of CTL tested for inhibition by peptides

CTL	Donor	Specificity of CTL	Target cell	Target molecule	A2.98-113	A2.94-112	Aw68.98-113	A2.56-69
line AJY	AK	A2	JY	A2	+	+	-	-
line PJY	PP	A2	JY	A2	+	+	-	-
clone A20.1	AK	A2	JY	A2	+	+	-	-
clone AI.10	AK	A2	JY	A2	+	+	-	-
clone AI9.1*	AK	A2, Aw68, Aw69	JY	A2	+	+	-	-
line PWSB	PW	A2, B17	JY	A2	+	+	-	-
line PWSB	PW	A2, B17	FMB	B17	-	-	-	-
clone AL8.1	AK	Aw68, Aw69	LB	Aw68	-	-	-	ND
clone AI5.1	AK	Aw69	IDF	Aw69	-	-	-	ND
line CJY	CC	Dr6	JY	DR6	-	ND	-	-
line CJY	CC	Dr6	DAUDI	DR6	-	ND	-	-

The specificity of the CTL is based upon analysis of the pattern of killing on a panel of B lymphoblastoid cell lines and by patterns of inhibition with monoclonal antibodies. The only restriction on CTL killing are the molecules listed under 'Specificity of CTL'. ND indicates not done. CTL used were from four donors with the following HLA types: AK (A3; B7, w38; C-; DR6), PP (A1, 3; B7, w62; Cw3; DR4), CC (A2, 26; Bw50; Cw6), PW (A2; Bw46, Cw1,3). All MHC class I-specific CTL were inhibited by monoclonal antibodies against monomorphic (PA2.6, W6/32) and appropriate polymorphic (PA2.1, MA2.1, CR11-351 for A2) determinants of the class I heavy chain. No inhibition was seen with anti- β_2 -microglobulin (BBM.1). The MHC class II-specific CTL were inhibited by monoclonal antibodies against monomorphic epitopes of HLA-DR (Ca.206) but not anti-class I³⁴.

* The observation that clone AI9.1 has specificity for both A2 and Aw68, but is only inhibited by the HLA-A2 peptide A2.98-113 and not the HLA-Aw68 peptide may result from the involvement of two regions; 98-113 and 140-160 in the CTL-target interaction. It is necessary to postulate that the interaction in the 98-113 region is stronger for A2 than Aw68 and this could be compensated by a stronger interaction with Aw68 in the 140-160 region. In this situation equivalent lysis of A2 and Aw68 targets would be associated with a more potent inhibition by peptide A2.98-113 over peptide Aw68.98-113. In this regard it should be noted that the only amino acids (threonine 142 and histidine 145), and monoclonal antibody-defined epitopes, that are specific to A2, Aw68 and Aw69 are found in the region 140-160.

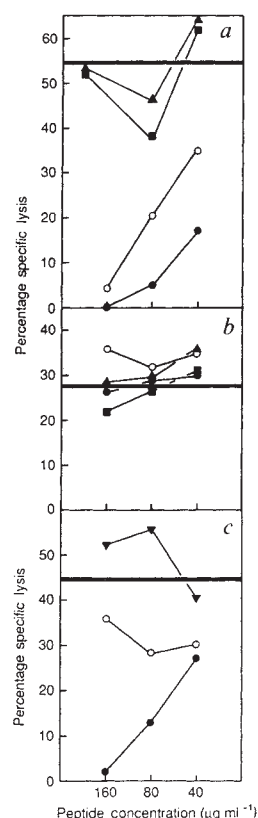


Fig. 2 *a, b*, Inhibition of HLA-A2 specific cytotoxic T cells (CTL) by peptides; *c*, destruction of inhibition by proteolysis. *a, b*, Peptide preparations were preincubated for 30 min with $1-3 \times 10^3$ CTL before addition of 10^3 ^{51}Cr -labelled B lymphoblastoid target cells. The peptides were present at the concentrations indicated throughout the cytotoxicity assay, which was performed as described^{24,37}. In *a* the CTL is AJY, a long term CD8^+ cell line specific for HLA-A2 and the target cell is the B lymphoblastoid cell line JY (HLA-A2, B7). In *b* the CTL is PWSB (a bulk culture with reactivity against HLA-B17) and the target is FMB, which expresses HLA-A1, A32, B17, 44. In each case the percentage specific release obtained in the absence of peptide is given by the bold horizontal line. The lower amount of specific release in panel *b* potentially made cytotoxicity more sensitive to inhibition. Peptides are: ●, A2.98-113; ○, A2.94-112; ▲, Aw68.98-113; ■, A2.56-69. Stocks of peptides at 1 mg ml^{-1} in phosphate-buffered saline (PBS) were diluted to give final concentrations in the assay as indicated. The final molar concentrations used in the assay at the middle dilution were $3.9 \times 10^{-5} \text{ M}$ for A2.98-113, $3.6 \times 10^{-5} \text{ M}$ for A2.94-112, $4.2 \times 10^{-5} \text{ M}$ for Aw68.98-113 and $4.7 \times 10^{-5} \text{ M}$ for A2.56-69. As a control inhibitor the monoclonal antibody PA2.6, which is directed against a monomorphic determinant of HLA-A,B,C molecules, was used^{37,40}. *c*, Proteolytic degradation destroys the inhibition of A2 specific CTL by HLA-A2 derived peptides. Aliquots of 1 mg ml^{-1} peptides in PBS or PBS alone were incubated for 16 h at 37°C with various proteinases or buffer at $25 \mu\text{g ml}^{-1}$. An additional and equivalent amount of proteinase was added after 6 h of incubation. The reactions were stopped by addition of 100 mM phenylmethylsulphonyl fluoride (PMSF) in 100% ethanol to a final concentration of 0.3 mM . These preparations were used as inhibitors of the CTL assay as described above. CTL used were an HLA-A2-specific clone designated AJ20.1 that derives from an HLA-A2-specific CTL line. The target is the JY lymphoblastoid B-cell line (HLA-A2; B7). Results shown are ●, A2.98-113 treated with buffer; ○, A2.98-113 treated with proteinase K; ▼, Aw68.98-113 treated with buffer. Incubation with trypsin and elastase had no effect on the inhibitory capacity of any peptide and chymotrypsin gave results that were similar to proteinase K. Controls included incubations with proteinases alone and with PMSF alone and these preparations did not affect the CTL assay. For each sample the degradation was monitored by HPLC on a C3 reverse phase column and was judged to be complete.

mutants in mouse^{3,14} and of HLA-A2^{15,16}, A3¹⁷, B7¹⁸ and B27¹⁹ variants in man have focused attention on residues 147-157 in the second extracellular domain of the class I heavy chain, although other substitutions can produce functional differences²⁰. With this approach direct identification of the parts of the class I molecule that interact with CTL receptors is not possible, for it does not distinguish substitutions within such sites from those located in other regions that affect conformation. To address this question we have examined whether synthetic peptides derived from the HLA-A2 sequence²¹ interfere with the activity of alloreactive CTL.

Sequence comparisons of HLA-A,B,C molecules show clusters of variability at the carboxy-terminal end (residues 62-83) of the α_1 domain and at the amino-terminal end (residues 94-116) of the α_2 domain (ref. 22; Fig. 1*a*). We therefore asked whether synthetic peptides from these two regions could affect cytotoxicity by HLA-A2-specific CTL (Fig. 1*b*). When a CTL line with specificity for HLA-A2 was preincubated with either of two peptides derived from residues 94-113 of the α_2 domain a significant and titratable inhibition of cytotoxicity was observed (Fig. 2*a*). The specificity of this inhibition was demonstrated in three ways. First, the peptide derived from residues 56-69 of the α_1 domain showed no inhibition, indicating that the inhibitory effect was specific to the sequence of the peptide. Second, we compared the inhibitory capacity of a peptide derived from residues 98-113 of the sequence of HLA-Aw68 with that of its HLA-A2 homologue. Within this region the two sequences only differ at position 107, where A2 has tryptophan and Aw68 has glycine²³. The Aw68.98-113 peptide does not inhibit A2-specific CTL (Fig. 2*a*), providing further evidence for the specificity of the inhibition by peptide A2.98-113 and showing that tryptophan 107 is necessary for this activity. Third, we considered the possibility that this tryptophan might endow the peptide in a non-specific way with properties that would enable it to inhibit all CTL. To test this we measured the effects of the peptides on various clones and lines of CTL with different target specificity. The results (Fig. 2*a, b*; summarized in Table 1) clearly show that only CTL which are specific for HLA-A2 are inhibited by peptide A2.98-113. CTL specific for other HLA class I or class II molecules were not inhibited.

Particularly informative results were obtained with CTL clones previously characterized in analysis of HLA-Aw69²⁴, a recombinant molecule having an α_1 domain identical to HLA-Aw68 and an α_2 domain identical to HLA-A2²³. Clones specific for HLA-A2 (A20.1, AI.10) and directed primarily to α_2 are inhibited by peptide A2.98-113 whereas clone AL8.1 specific for both HLA-Aw68 and HLA-Aw69 and directed primarily against α_1 is not inhibited (Table 1). Neither was clone AI5.1, which is specific for HLA-Aw69 and dependent upon a combination of the α_1 and α_2 domains, inhibited. This analysis strongly argues for the inhibition being related to the specificity of the CTL and by inference to the specificity of their T-cell receptors.

We next examined the effects of proteolytic digestion on the inhibitory capacity of the peptides. Digestion with proteinase K (Fig. 2*c*) and chymotrypsin reduced the inhibitory activity. In contrast no effect was seen with trypsin or elastase although HPLC analysis indicated that significant cleavage had occurred. Thus smaller peptides may be as potent inhibitors as A2.98-113. It may therefore be relevant that the sequences between residues 105 and 108 of all class I molecules are related to that of the fibronectin binding tetrapeptide²⁵. This peptide RGDS and its reverse SDGR both have cell attachment properties^{26,27}. HLA-Aw68 contain a perfect SDGR whereas in HLA-A2 the tryptophan breaks this sequence.

Our results show that residues 98-113 form an epitope recognized by the receptors of a significant proportion of the CTL stimulated by HLA-A2. This is a different part of the α_2 domain than the region from 147-157 frequently implicated from the sequences of class I mutants and variants^{2-7,15-20}. These results are not incompatible as different regions may be involved,

depending on CTL and the specific class I target molecule. These regions may also be adjacent in the three-dimensional structure, with both contributing to structures recognized by a single CTL. That a substitution at either position 106 or at 161 eliminates a single monoclonal-antibody-defined epitope of HLA-A2 supports this hypothesis^{28,29}. The involvement of two regions, residues 98–113 and 140–160, in interaction with CTL could explain why clone AI9.1, which is specific for A2, Aw68 and Aw69 is inhibited by the HLA-A2 peptide A2.98–113 and not the HLA-Aw68 peptide Aw68.98–113 (see Table 1).

The tryptophan at position 107 that is important in the epitope seen by CTL is only found in subtypes of HLA-A2 and HLA-Aw69; all other HLA-A,B,C molecules so far analysed have glycine at this position²². Thus this position is one in which there is only a limited variation and in fact the region covered by peptide 98–113 contains no position at which more than two different amino acids have been found. Interestingly it is flanked by two pairs of residues (96, 98 and 114, 116) at which considerable variation occurs²² (Fig. 1a).

HLA-A2 was one of the first HLA specificities to be defined and continues to be a major focus of research³⁰. Three variant subtypes of HLA-A2 are distinguished by CTL and differ from the predominant form by 1–4 amino acid substitutions^{15,16,20}. In addition a second group of HLA-Aw68 related molecules can be included in the HLA-A2 family²³. Sequence comparisons within this family have enabled us to identify residues responsible for epitopes defined by monoclonal antibodies³¹. Residues 62–65 are involved in the MA2.1 epitope shared by HLA-A2 and HLA-B17 and tryptophan 107 is responsible for the PA2.1 epitope shared by HLA-A2 and HLA-Aw69^{20,21,32}. But it has not been possible to demonstrate binding or inhibition of the monoclonal antibodies with peptides derived from these regions, even at concentrations far in excess of those that inhibit CTL. This is consistent with reports that the HLA-A,B,C epitopes recognized by alloantiserum, monoclonal antibodies and polyclonal xenoantisera are dependent upon conformation^{33–35}. Here we show that, in contrast to the experience with antibodies, an interaction between short peptides and HLA-A2-specific CTL can be demonstrated. This distinction raises the possibility that antigen receptors of alloreactive T cells preferentially recognize degraded fragments of class I molecules. This would explain why the determinants recognized by antibodies and CTL often seem quite different¹¹. A role for intact molecules would still be required to explain the potent blocking of CTL by monoclonal antibodies against conformationally dependent determinants^{36,37}. It is, however, possible that both degraded and intact class I molecules are involved. This idea is attractive as alloreactive CTL recognition then becomes a special case of MHC restriction in which degraded class I molecules assume the role of nominal antigen and intact class I molecules are the restriction elements.

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1. Zinkernagel, R. M. & Doherty, P. C. *Adv. Immun.* **27**, 51–177 (1979).
2. Duran, L. W. & Pease, L. R. *Transplantation* **41**, 279–285 (1986).
3. Nathenson, S. G., Geliebter, J., Pfaffenbach, G. M. & Zeff, R. A. *Rev. Immun.* **4**, 471–502 (1986).
4. Lopez de Castro, J. A., Barbosa, J. A., Krangel, M. S., Biro, P. A. & Strominger, J. L. *Immun. Rev.* **85**, 149–168 (1985).
5. Jordan, B. R. *et al. Immun. Rev.* **84**, 73–92 (1985).
6. Lew, A. M. *et al. Immunology* **57**, 3–18 (1986).
7. Townsend, A. R. M. & McMichael, A. J. in *Progress in Allergy* Vol. 36 (eds de Vries, R. R. P. & van Rood, J. J.) 10–43 (Karger, Basel, 1985).
8. Lindahl, K. F. & Bach, F. H. *Nature* **254**, 607–609 (1975).
9. Nabholz, M. *et al. Eur. J. Immun.* **4**, 378–387 (1974).
10. Townsend, A. R. M. *et al. Cell* **44**, 959–968 (1986).
11. Biddison, W. E., Ward, F. E., Shearer, G. M. & Shaw, S. J. *Immun.* **124**, 548–552 (1980).

12. Spits, H., Breuning, M., Ivanyi, P., Russo, C. & de Vries, J. E. *Immunogenetics* **16**, 503–512 (1982).
13. Gaston, J. S. H., Rickinson, A. B. & Epstein, M. A. *J. exp. Med.* **158**, 280–293 (1983).
14. Schulze, D. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 2007–2011 (1983).
15. Krangel, M. S., Taketani, S., Biddison, W. E., Strong, D. M. & Strominger, J. L. *Biochemistry* **21**, 6313–6321 (1982).
16. Krangel, M. S., Biddison, W. E. & Strominger, J. L. *J. Immun.* **130**, 1856–1862 (1983).
17. Cowan, E. P., Jordan, B. R. & Coligan, J. E. *J. Immun.* **135**, 2835–2841 (1985).
18. Taketani, S., Krangel, M. S., Spits, H., de Vries, J. & Strominger, J. L. *J. Immun.* **133**, 816–821 (1984).
19. Vega, M. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 7394–7398 (1985).
20. Ezquerro, A. *et al. J. Immun.* **137**, 1642–1649 (1986).
21. Koller, B. H. & Orr, H. T. *J. Immun.* **134**, 2727–2733 (1985).
22. Ways, J. P., Coppin, H. L. & Parham, P. *J. biol. Chem.* **260**, 11924–11933 (1985).
23. Holmes, N. & Parham, P. *EMBO J.* **4**, 2849–2854 (1985).
24. Clayberger, C. *et al. J. exp. Med.* **162**, 1709–1714 (1985).
25. Auffray, C. & Novotny, J. *Hum. Immun.* **15**, 381–390 (1986).
26. Pierschbacher, M. D. & Ruoslahti, E. *Nature* **309**, 30–33 (1984).
27. Yamada, K. M., Kennedy, D. W. *J. cell. Biochem.* **28**, 99–104 (1985).
28. Krangel, M. S., Taketani, S., Pious, D. & Strominger, J. L. *J. exp. Med.* **157**, 324–336 (1983).
29. Taketani, S., Krangel, M. S., Pious, D. & Strominger, J. L. *J. Immun.* **131**, 2935–2938 (1983).
30. Dausset, J., Ivanyi, P. & Ivanyi, D. in *Histocompatibility Testing 1965* (eds, Balner, H., Cleton, F. L. & Eernisse, J. G.) 51–62 (Munksgaard, Copenhagen, 1965).
31. Ways, J. P. & Parham, P. *Biochem. J.* **216**, 423–432 (1983).
32. Ways, J. P., Rothbard, J. B. & Parham, P. *J. Immun.* **137**, 217–222 (1986).
33. Lancet, D., Parham, P. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3844–3848 (1979).
34. Parham, P., Androlewicz, M. J., Brodsky, F. M., Holmes, N. J. & Ways, J. P. *J. immun. Meth.* **53**, 133–173 (1982).
35. Krangel, M. S., Orr, H. T. & Strominger, J. L. *Cell* **18**, 979–991 (1979).
36. Lindahl, F. K. & Lemke, H. *Eur. J. Immun.* **9**, 526–536 (1979).
37. Reiss, C. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 5432–5436 (1980).
38. Erickson, B. W. & Merrifield, R. B. in: *The Proteins* Vol. 2, 3rd edn (eds Neurath, H. & Hill, R. L.) 255–527 (Academic, New York, 1970).
39. Wan, A. M., Ennis, P., Parham, P. & Holmes, N. J. *Immun.* **137**, 3671–3674 (1986).
40. McMichael, A. J., Parham, P., Brodsky, F. M. & Pilch, J. R. *J. exp. Med.* **152**, 195s–203s (1980).

Pleiotropic loss of activation pathways in a T-cell receptor α -chain deletion variant of a cytolytic T-cell clone

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Untransformed T-cell clones maintained in culture are dependent on signals transmitted through their antigen receptors (Ti; α and β chains associated with the CD3 molecules) for growth and effector function^{1,2}. For cytolytic T cells (CTL), Ti stimulation also activates the killing machinery³ and induces synthesis of gamma interferon (IFN- γ) messenger RNA and IFN- γ secretion⁴. The Thy-1 molecule, expressed on all murine cells of the T-cell lineage, has been suggested to function in transmembrane signalling, based on the ability of some anti-Thy-1 monoclonal antibodies (mAb) to activate T cells^{5–9}. Recently, it was suggested that Thy-1 could function as a signal-transduction molecule when expressed in B-cell lymphomas after transfection of the gene, leading to speculation that the molecule was part of an activation pathway independent of the Ti/CD3 structures¹⁰. Here we report the immunoselection of a variant CTL clone which has lost expression of mRNA for the α -chain of the Ti. The Ti[−] variant was defective in lectin-mediated activation whether measured by increase in intracytoplasmic Ca²⁺, CTL effector function or IFN- γ synthesis. The variant, which expressed normal levels of Thy-1, was also unresponsive to Thy-1 mAb activation as measured by IFN- γ secretion, whereas it responded to calcium ionophore plus phorbol ester. These results indicate that in a non-transformed, functional mature T-cell, Thy-1 mediated signalling is not an alternative to, but might depend on elements associated with the Ti/CD3-mediated T-cell activation pathway.

Fig. 1 Negative selection using MTX-containing protein A-coupled liposomes (MTX-lip-PA) was adapted from Machy and Leserman¹³. To 10^6 KB5-C20 cells cultured for 24 h in 1 ml 10% interleukin-2(IL-2)-containing supernatant from PMA-stimulated EL4.C16 cells¹¹ (SC16) were added anti-Ti mAb Désiré-1 (ref. 12) ($1 \mu\text{g ml}^{-1}$) and MTX-lip-PA (final MTX concentration of 60 nM). After 24 h, the medium was replaced with 10% SC16 and a second treatment with MTX-lip-PA was performed 3 days later. Most cells had died after the two treatments and visible growth resumed after 2 weeks. A 2-h activation step using $1 \mu\text{M}$ ionomycin (Calbiochem) and 1.6×10^{-9} M PMA (Sigma) (for conditions see ref. 11) followed by replacement of the medium with 10% SC16 was performed at that time. One week later, cells were collected and transferred at 10^5 cells ml^{-1} in the presence of 10^6 2,500-rad irradiated CBA/J splenic feeder cells and 10% SC16. For immunofluorescence, T cells (2×10^5), collected 3 days after restimulation with either ionomycin/PMA and 10% SC16 (KB5-C20*, KB5-C20.lip.A.2*) or with irradiated C57BL/6 spleen cells and 10% SC16 (KB5-C20, BM3.3), were incubated in round-bottom microtitre plates for 30 min at 4°C with $40 \mu\text{l}$ mAb at $10 \mu\text{g ml}^{-1}$. Clone BM3.3 was used as a control, being a H-2K^b-specific CTL clone of CBA/J origin (Lyt-2.1) which does not express the Désiré-1 clonotype¹². After 2 washes, cells were incubated with $20 \mu\text{l}$ carboxyfluorescein-containing PA-liposomes for 1 h at 4°C , washed and fluorescence was recorded with a FACS for 10^4 cells. MAbs were Désiré-1 (anti-Ti of KB5-C20)¹², anti-Lyt-2.1 (NE1-004) and anti-Lyt-2.2 (19.178), which all bound protein A. Control samples were incubated with the protein A-binding mAb 20.8.4 (which does not bind to the H-2^k CTL clones (left curves)). Staining for expression of Thy-1 was done in one step with fluorescein-labelled anti-Thy-1.2 mAb 30.H12 (ref. 15) for 30 min at 4°C , using a fluorescein-labelled anti-mouse IgM as a control.

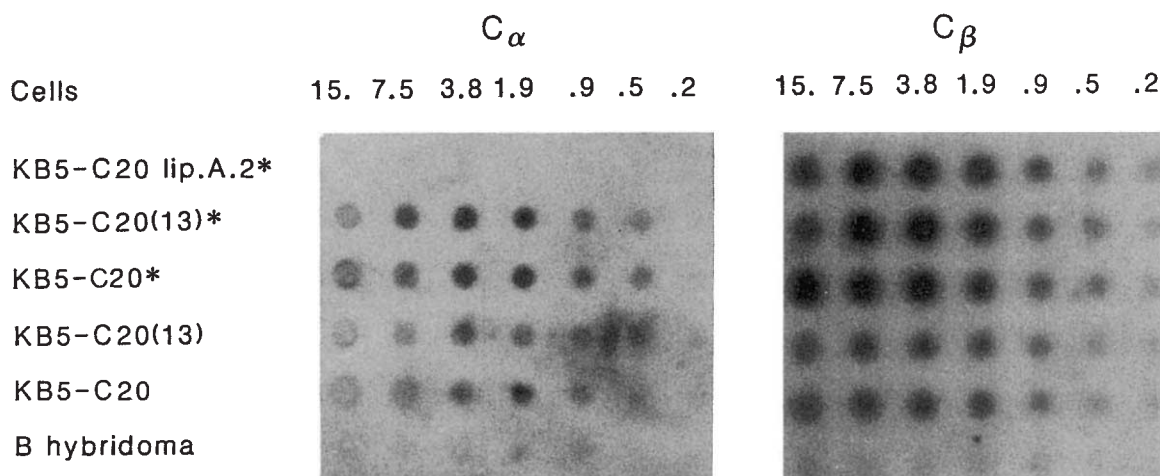
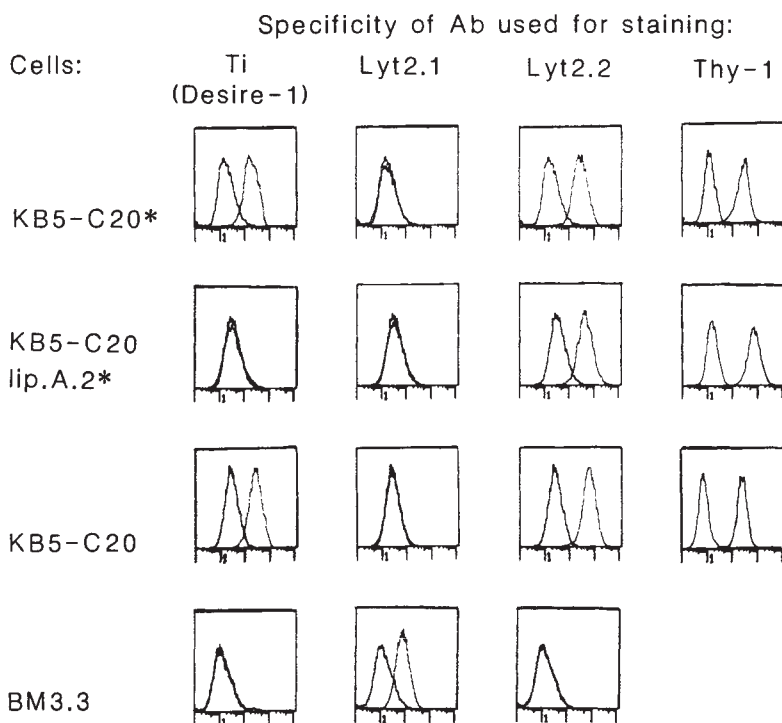


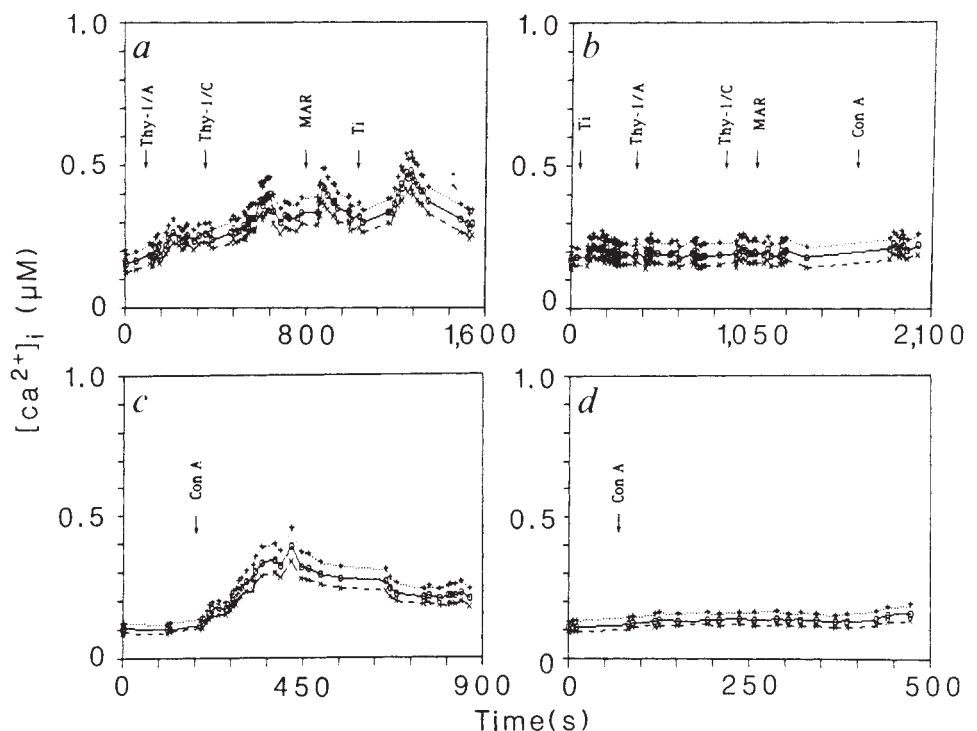
Fig. 2 RNA dot blots were prepared as described²⁴. Three days after their last restimulation with ionomycin/PMA and IL-2-containing SC16 (KB5-C20 lip.A.2*, KB5-C20(13)* (a subclone of KB5-C20), clone KB5-C20*), or with irradiated C57BL/6 spleen cells and IL-2-containing SC16 (subclone KB5-C20(13) and clone KB5-C20), T-cell clones were collected on ice and washed in cold PBS. Cells from a B-cell hybridoma (100.5.28) grown in culture were used as a control. Serial dilutions of lysates, corresponding to the indicated number of cells, containing formaldehyde-denatured cytoplasmic RNA were applied to nitrocellulose filters as described²⁴. C_α and C_β cDNA probes corresponded respectively to 493 and 434 base pairs of the C fragments of the genes coding for the α and β chain of the T-cell receptor. The cDNAs were labelled with ^{32}P -dCTP by the hexamer priming technique, as described²⁵. After prehybridization, nitrocellulose filters were hybridized with about 10^9 c.p.m. of ^{32}P -labelled cDNA probe (100 ng DNA) as described²⁴ and exposed to XAR5 film for 48 h. For successive hybridizations of the same filter, the ^{32}P -probe was washed off the filters by 30 min boiling in water.

We used the Ca^{2+} ionophore ionomycin and the phorbol ester phorbol myristate acetate (PMA) as substitutes for Ti-mediated activation¹¹, combined with immunoselection with the anti-clonotypic (anti-Ti) mAb Désiré-1¹² to isolate a CTL variant (called KB5-C20.lip.A.2) lacking surface expression of the Ti from the H-2K^b-specific clone KB5-C20 (Fig. 1). The variant was selected with methotrexate (MTX)-containing, protein A-coupled liposomes targeted to the T-cell receptor via the anti-Ti mAb using a previously described technique¹³. Northern dot blot analysis indicated that the variant lacked mRNA for the

α -chain of the Ti (Fig. 2). Other cell surface markers such as Lyt-2.2, and Thy-1 (Fig. 1) or H-2K^k (results not shown) were expressed at similar levels on clone KB5-C20 and on variant KB5-C20.lip.A.2.

The availability of variant KB5-C20.lip.A.2 allowed the analysis of the influence of lack of Ti expression (and of the associated CD3 molecules (not shown)) on CTL effector functions. Results in Table 1 indicate that KB5-C20.lip.A.2 no longer lysed the H-2K^b-expressing target cell EL4.BU, which was efficiently lysed by the KB5-C20 cells (Table 1a). The variant also lost the

Fig. 3 Measure of intracytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). KB5-C20 (a, c) and KB5-C20.lip.A.2* (b, d) cells were loaded with the acetoxymethyl ester form of the Ca^{2+} -sensitive dye Fura-2²⁶ (1 ml 1 μM Fura-2 (Molecular Probes) for 10^6 cells in RPM1+5% FCS for 30 min at 37°C), washed and resuspended in 0.3 ml Hank's balanced salt solution (HBSS) containing 1 mM Ca^{2+} and 3% FCS. Cells were left to settle on a poly-L-lysine-coated glass cover slip placed in a 37°C heated chamber on a fluorescence microscope as described³. Fluorescence was recorded on single cells at 350 and 385 nm (as described in ref. 3) at each indicated time point before and after sequential addition of mAb as indicated: Ti, 30 $\mu\text{g ml}^{-1}$ mAb Désiré-1; Thy-1/A, 30 $\mu\text{g ml}^{-1}$ mAb H129.93.9.1; Thy-1/C, 30 $\mu\text{g ml}^{-1}$ anti-Thy-1 mAb H155.124.3; MAR, 30 $\mu\text{g ml}^{-1}$ mouse anti-rat κ mAb; Con A, 5 $\mu\text{g ml}^{-1}$ ConA. The increase in $[\text{Ca}^{2+}]_i$ was measured for individual cells by the increase in the ratio of 350-nm to 385-nm excitation efficiency of Fura-2, which was quantified by a video silicon-intensified camera and digital image processing as described^{3,27}. Mean $[\text{Ca}^{2+}]_i$ (○) was plotted as a function of time for about 50 cells. The mean was calculated with cells weighted according to their average intensity. The upper (+) and lower (×) error estimates were the weighted means of all the cells' individual upper and lower bounds in $[\text{Ca}^{2+}]_i$. Addition of ionomycin (5 μM) produced $[\text{Ca}^{2+}]_i > 1.5 \mu\text{M}$ in all samples (not shown).



capacity to mediate antigen-independent, lectin-dependent cell-mediated cytotoxicity (LDCC) as indicated by lack of lytic activity on RDM4 target cells (Table 1b) in the presence of Concanavalin A (Con A), as compared to the ConA-dependent lysis exerted by KB5-C20. Similar results were obtained when phytohemagglutinin (PHA) was used to measure LDCC (results not shown). Although participation of the Ti in LDCC had previously been suggested on the basis of blocking of LDCC by one anti-Ti mAb¹⁴, the present results clearly establish the requirement for Ti/CD3 expression for LDCC.

To analyse whether the requirement for Ti expression was specific for the activation for killing, induction of IFN- γ secretion was used as another means of measuring activation. Results in Table 1c indicate that, as previously described¹¹, clone KB5-C20 was induced to secrete IFN- γ , as measured by macrophage-activating factor (MAF) activity, in response to either C57BL/6 cells, Con A or a combination of ionomycin and PMA. KB5-C20.lip.A.2 retained the capacity to make IFN- γ in response to ionomycin/PMA, but lost responsiveness not only to C57BL/6, but also to Con A. The basis for the enhanced efficiency of stimulation of clone KB5-C20.lip.A.2 with ionomycin alone, as compared to the requirement of both ionomycin and PMA for the stimulation of the Ti⁺ clones is as yet unclear.

Binding of mAb to certain epitopes of the Thy-1 molecule has recently been shown to induce the activation programme of T-cell hybridomas^{6,7} and CTL⁸. In particular, Pont *et al.*⁷ described rat mAb directed against three distinct epitopes of the Thy-1 molecule such that anti-Thy-1/C and anti-Thy-1/A mAb were stimulatory in the absence and presence, respectively, of cross-linking with mouse anti-rat κ mAb (MAR), whereas anti-Thy-1/B mAb were not stimulatory. We extended these observations to CTL clones⁹. Table 1c shows that mAb H155.124.3 (Thy-1/C) could induce IFN- γ secretion from clone KB5-C20 but not from variant KB5-C20.lip.A.2. The monoclonal antibody H140.61 (Thy-1/B) was not stimulatory for either clone. Lack of responsiveness to anti-Thy-1 mAb cannot be

attributed to absence of surface Thy-1, as detected by mAb 30.H12 (ref 15) (Fig. 1) or by mAb H155.124.3 (results not shown).

Because Ti¹⁶, Con A¹⁷ and Thy-1¹⁰-mediated activation have in common an initial increase in internal calcium concentration $[\text{Ca}^{2+}]_i$, we analysed this parameter on the Ti-negative variant. Results in Fig. 3a indicate that clone KB5-C20 responded with an increase in $[\text{Ca}^{2+}]_i$ after addition of anti-Thy-1 mAb H129.93.9.1 (Thy-1/A) and H155.124.3 (Thy-1/C) followed by MAR. These mAb have been shown to act synergistically in T-cell activation⁷. Similar results were obtained with clone KB5-C20* (maintained in culture by stimulation with ionomycin and PMA in the same manner as clone KB5-C20.lip.A.2*) (results not shown). Both Ti⁺ cells also increased their $[\text{Ca}^{2+}]_i$ in response to anti-Ti mAb Désiré-1 (Fig. 3a) and the response was further increased after addition of a rabbit anti-mouse immunoglobulin reagent (result not shown). No change in $[\text{Ca}^{2+}]_i$ was observed on binding of rat anti-Lyt-2 mAb (53.6.7) followed by MAR to either KB5-C20, KB5-C20* or KB5-C20.lip.A.2* (results not shown). ConA alone induced an increase in $[\text{Ca}^{2+}]_i$ in the two Ti⁺ KB5-C20 clones (Fig. 3c and results not shown). The Ti⁻ clone KB5-C20.lip.A.2, however, was totally unresponsive, as measured by $[\text{Ca}^{2+}]_i$, to the Désiré-1 anti-Ti mAb, the combination of anti-Thy-1 mAb followed by MAR (Fig. 3b), and also to ConA (Fig. 3b,d).

The simultaneous loss of expression of the Ti structure and of activation induced by either Thy-1-specific mAb or ConA was also observed for sub-clones derived from KB5-C20.lip.A.2 as well as for three other variants obtained independently with the same immunoselection technique (not shown), whereas Ti⁺ subclones of KB5-C20 were always found to be responsive. The recurrence of the observation strongly suggests that there is a pleiotropic loss in activation pathways rather than a concomitant, random selection for loss of Ti expression and loss of Con A and Thy-1-mediated activation pathways. Attempts at re-expressing the α -chain mRNA after transfection of the gene are

Table 1 Characteristics of the Ti⁻ variant KB5-C20.lip.A.2

a, H-2K ^b -specific CTL activity measured on EL4 target cells:						
E:T	KB5-C20		KB5-C20*		KB5-C20.lip.A.2*	
5:1	52		25		0	
1:1	25		10		0	

b, Con A-induced CTL activity measured on RDM4 target cells:						
E:T	KB5-C20		KB5-C20*		KB5-C20.lip.A.2*	
	-ConA	+ConA	-ConA	+ConA	-ConA	+ConA
10:1	-0.2	46	-0.4	29	2.2	6.0
3:1	0.0	28	1.6	28	2.6	2.0
1:1	-0.3	12	0.8	15	1.5	2.6
0.3:1	1.0	6.0	-0.5	10	-0.7	0.2

c, MAF units in supernatants from CTL clones:				
	KB5-C20		KB5-C20.lip.A.2*	
Stimulated with	-PMA	+PMA	-PMA	+PMA
Control	<3	<3	<3	<3
Ionomycin	<3	50	55	290
CBA spleen cells	<3	<3	<3	<3
C57BL/6 spleen cells	74	77	<3	<3
Anti-Ti mAb	54	118	<3	<3
Anti-Thy-1/C mAb	300	160	<3	<3
Anti-Thy-1/B mAb	<3	<3	<3	<3
Concanavalin A	240	150	<3	<3

In *a* and *b*, cytolytic activities of clones KB5-C20, KB5-C20* and KB5-C20.lip.A.2* were measured in a 5 h ⁵¹Cr-release assay as described¹² on the H-2^b EL4.BU (*a*) or on the H-2^k RDM4 (*b*) target cells untreated for direct lysis or preincubated for 30 min at 37 °C with 10 µg ml⁻¹ Con A for lectin-dependent lysis (LDCC). The percentage specific ⁵¹Cr release (mean of triplicate samples) from 10⁴ target cells is shown for different effector to target cell ratios (E:T). In *c*, macrophage activating factor (MAF) was measured as described¹¹ in supernatants from 10⁵ clone cells incubated for 20 h at 37 °C in 96-well flat-bottomed microtitre plates in 0.2 ml medium in the absence (-PMA) or in the presence (+PMA) of 1.6 × 10⁻⁹ M PMA. In all conditions of stimulation, MAF was a measure of interferon gamma (IFN-γ) as indicated by inhibition of MAF activity with IFN-γ specific mAb (A.G., C.B. and M.B., unpublished results). Stimulation conditions of the CTL clones were as follows: ionomycin, 2 µM; spleen cells from CBA/J(H-2^k) or from C57BL/6(H-2^b) mice, 10⁶ cells irradiated at 2,000 rad; anti-Ti mAb: 30 µg ml⁻¹ Désiré-1¹²; anti-Thy-1/C mAb, 30 µg ml⁻¹ H155.124.3 (ref. 7); anti-Thy-1/B mAb, 30 µg ml⁻¹ H140.61.2 (ref. 7); ConA, 5 µg ml⁻¹ ConA.

in progress. The finding of lack of lectin responsiveness of the Ti⁻ variant extends to a non-transformed CTL the observation by Weiss and colleagues on the lack of PHA stimulation of Tiβ-chain-deficient Jurkat cells¹⁸, a property which could be recovered after transfection of the β-chain gene¹⁹. It furthermore indicates that among the different CTL cell surface glycoproteins which bind ConA²⁰, only the Ti complex effectively transduces intracellular signals, whether measured by early [Ca²⁺]_i, by CTL effector function, or by IFN-γ synthesis.

The striking result reported here is the apparent dependence of the Thy-1-mediated activation pathway on expression of the Ti complex. This is surprising in view of the fact that the Thy-1 molecule was not coprecipitated with the Ti-CD3 complex from clone KB5-C20 (ref. 12) and that the two molecules could be modulated independently from the cell surface⁹. There is not necessarily a contradiction between our results and the finding¹⁰ that the Thy-1-molecule expressed on a B lymphoma after gene transfer manifested an increase in [Ca²⁺]_i in response to activating Thy-1-specific mAb. That experiment does not prove the independence of the Thy-1-mediated signalling pathway as surface immunoglobulin or associated structures on B cells might replace the function of the Ti-CD3 complex on T cells. Using human T cells, O'Flynn and colleagues²¹ suggested that PHA activation was mediated by the CD2-dependent pathway. The latter conclusion, being based only on mAb blocking data using cells also expressing the Ti-CD3 complex, is probably not definitive. However, a subpopulation of human thymocytes expressing the CD2 antigen in the absence of Ti/CD3 surface expression appears to be stimulated through the CD2 pathway²². No equivalent to the human CD2 molecule is known in the mouse, but a similar distribution on immature and mature T cells suggested that in the mouse the Thy-1 molecule might be part of a signalling pathway distinct from the Ti-mediated path-

way and possibly used for activation of cells of the T lineage before surface expression of the Ti complex¹⁰. Results presented here clearly show that on mature functional T cells, the Thy-1 structure does not function as an alternative activation pathway to the Ti complex. It remains to be determined whether the dependence of Thy-1-mediated activation on the Ti/CD3 complex is at the level of the generation of inositol trisphosphate leading to Ca²⁺ mobilization²³ or at the level of Ca²⁺ influx through a channel possibly associated with the Ti complex.

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- Robb, R. J., Munck, A. & Smith, K. A. *J. exp. Med.* **154**, 1455-1474 (1981).
- Meuer, S. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 1509-1513 (1984).
- Poenie, M., Tsien, R. Y. & Schmitt-Verhulst, A.-M. *EMBO J.* (submitted).
- Moldwin, R. L., Lancki, D. W., Herold, K. C. & Fitch, F. W. *J. exp. Med.* **163**, 1566-1582 (1986).
- Konata, Y., Norcross, M. A., Maino, V. C. & Smith, R. T. *Eur. J. Immun.* **11**, 445-449 (1981).
- Gunter, K. C., Malek, T. R. & Shevach, E. M. *J. exp. Med.* **159**, 716-730 (1984).
- Pont, S. *et al. Eur. J. Immun.* **15**, 1222-1228 (1985).
- MacDonald, H. R., Bron, C., Rousseaux, M., Horvath, C. & Cerottini, J. C. *Eur. J. Immun.* **15**, 495-501 (1985).
- Guimezanes, A., Buferne, M., Pierres, M. & Schmitt-Verhulst, A.-M. (in preparation).
- Kroczeck, R. A., Gunter, K. C., Germain, R. N. & Shevach, E. M. *Nature* **322**, 181-184 (1986).
- Albert, F., Hua, C., Truneh, A., Pierres, M. & Schmitt-Verhulst, A.-M. *J. Immun.* **134**, 3649-3655 (1985).
- Hua, C., Boyer, C., Buferne, M. & Schmitt-Verhulst, A.-M. *J. Immun.* **136**, 1937-1944 (1986).
- Machy, P. & Leserman, L. D. *EMBO J.* **3**, 1971-1977 (1984).
- Hubbard, S. C., Kranz, D. M., Longmore, G. D., Sitkovsky, M. V. & Eisen, H. N. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1852-1856 (1986).
- Ledbetter, J. A. & Herzenberg, L. A. *Immun. Rev.* **47**, 63-90 (1979).
- Weiss, A., Imboden, J., Shoback, D. & Stobo, J. D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4169-4173 (1984).
- Tsien, R. Y., Pozzan, T. & Rink, T. J. *Nature* **295**, 68-71 (1982).
- Weiss, A. & Stobo, J. D. *J. exp. Med.* **160**, 1284-1299 (1984).
- Ohashi, P. S. *et al. Nature* **316**, 606-609 (1985).
- Sitkovsky, M. V., Pasternack, M. S., Lugo, J. P., Klein, J. & Eisen, H. N. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1519-1523 (1984).
- O'Flynn, K., Krensky, A. M., Beverley, P. C. L., Burakoff, S. J. & Linch, D. C. *Nature* **313**, 686-687 (1985).
- Fox, D. A. *et al. J. Immun.* **134**, 330-335 (1985).
- Nishizuka, Y. *Nature* **308**, 693-698 (1984).
- Meinkoth, J. & Wahl, G. *Analyt. Biochem.* **138**, 267-284 (1984).
- Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6-13 (1983).
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. *J. biol. Chem.* **260**, 3440-3450 (1985).
- Poenie, M., Alderton, J., Steinhardt, R. & Tsien, R. Y. *Science* **233**, 886-889 (1986).

A novel c-abl protein product in Philadelphia-positive acute lymphoblastic leukaemia

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Activation of cellular proto-oncogenes as a result of chromosomal abnormalities has been implicated in the development of some human malignancies. Perhaps one of the most striking examples of this association occurs in chronic myelogenous leukaemia, where the Philadelphia (Ph) translocation results in substitution of the 5' end of the c-abl proto-oncogene with bcr gene sequences. A unique hybrid bcr-abl message is produced. As the Ph translocation is also present in some patients with acute lymphoblastic leukaemia, we initiated studies to determine if similar genomic events occur in these two different forms of Ph-positive leukaemia. Here we report that the Ph translocation in acute lymphoblastic

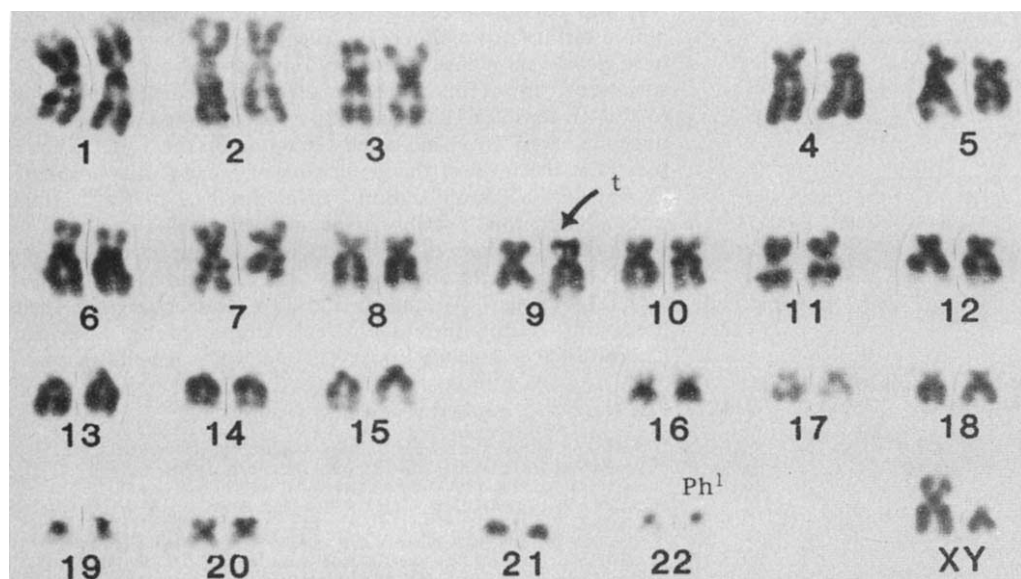


Fig. 1 Karyotype of Ph-positive acute lymphoblastic leukaemia patient 2. Giemsa banded chromosomes of the bone marrow cells show a pseudodiploid clone 46, XY, t(9;22)(q34;q11).

Methods. Standard cytogenetic procedures were used; slide preparations were stained with Giemsa stain after trypsin pretreatment, yielding G-banded chromosomes²⁹.

leukaemia can result in production of a novel aberrant *c-abl* protein that is distinct from the *bcr-abl* protein found in Ph-positive chronic myelogenous leukaemia. Our observations suggest that alternative mechanisms of activation of *c-abl* exist, and may be important in the development of human acute lymphoid rather than chronic myeloid malignancies.

In 1960, Nowell and Hungerford discovered a shortened chromosome 22, designated the Philadelphia (Ph) chromosome, in chronic myelogenous leukaemia (CML)¹. This chromosomal aberration, which is now considered the cytogenetic hallmark of the disease, results from a reciprocal translocation between chromosomes 9 and 22, [t(9;22)(q34;q11)]^{2,3}. The breakpoints on chromosome 22 occur within a 5.8 kilobase (kb) segment of DNA, termed the breakpoint cluster region or *bcr*^{4,5}. A crucial event in the Ph translocation is the transfer of the *c-abl* proto-oncogene from its normal residence on chromosome 9 to chromosome 22, so that *bcr* and *c-abl* sequences are juxtaposed in a head-to-tail configuration with *bcr* closer to the centromere⁶⁻⁸. The resulting mRNA is a hybrid of 5' *bcr* sequences and a truncated *c-abl* gene lacking the first exon^{8,9}. This chimaeric 8.5-kb *bcr-abl* transcript encodes an aberrant protein of relative molecular mass 210,000 (M_r 210K), whereas the normal *c-abl* gene is expressed as 6- and 7-kb messenger RNAs encoding a 145K protein¹⁰⁻¹³. Both p210^{*bcr-abl*} and its normal homologue, p145^{*c-abl*}, as well as the Abelson murine leukaemia virus-encoded p160^{*gag-abl*} have an associated tyrosine protein kinase activity. However, p160^{*gag-abl*} and p210^{*bcr-abl*} probably use themselves as substrates in a different way from p145^{*c-abl*} *in vitro*¹². The high specificity and altered autophosphorylation properties of p210^{*bcr-abl*} strongly imply that this molecule is critical in the pathogenesis of CML.

Interestingly, the Ph chromosome has also been detected in ~17-25% of adult patients with acute lymphoblastic leukaemia (ALL)^{14,15}. It is not known whether the presentation as acute leukaemia or CML represents a different clinical manifestation of the same malignant process—Ph positive leukaemia—or, despite the presence of an identical cytogenetic abnormality, a distinction should be drawn between the two disorders. A strong argument supporting a single underlying disease process for at least some cases of Ph-positive ALL and CML are the reports of patients presenting with ALL in whom transition to CML has been documented¹⁶. In addition, ~1/3 of patients with CML will eventually develop a lymphoid blast transformation¹⁷. This clinical state strongly resembles ALL. Nevertheless, these two disorders have fundamental differences, with the salient feature of CML being the rapid proliferation of mature myeloid cells

whereas that of ALL is a greatly increased number of immature lymphoid cells. These unique clinical manifestations of Ph-positive CML compared with ALL suggest discrete events in their pathogenesis.

As genetic events are assumed to be critical determinants of the phenotypic manifestations of disease, we have studied *c-abl* expression and searched for *bcr* rearrangement in two Ph-positive ALL patients. The diagnosis of ALL was based on several features: (1) presentation with >80% blasts in a hypercellular bone marrow; (2) lymphoblastoid-appearing cells by light microscopy; and (3) positive terminal deoxynucleotidyl trans-

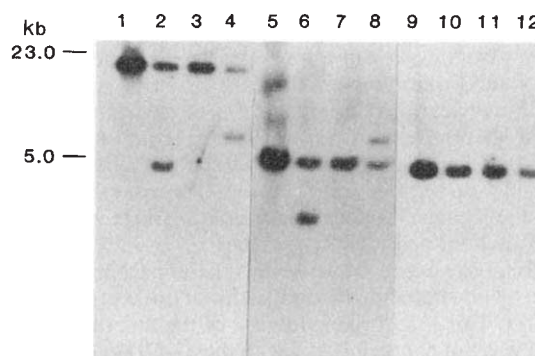


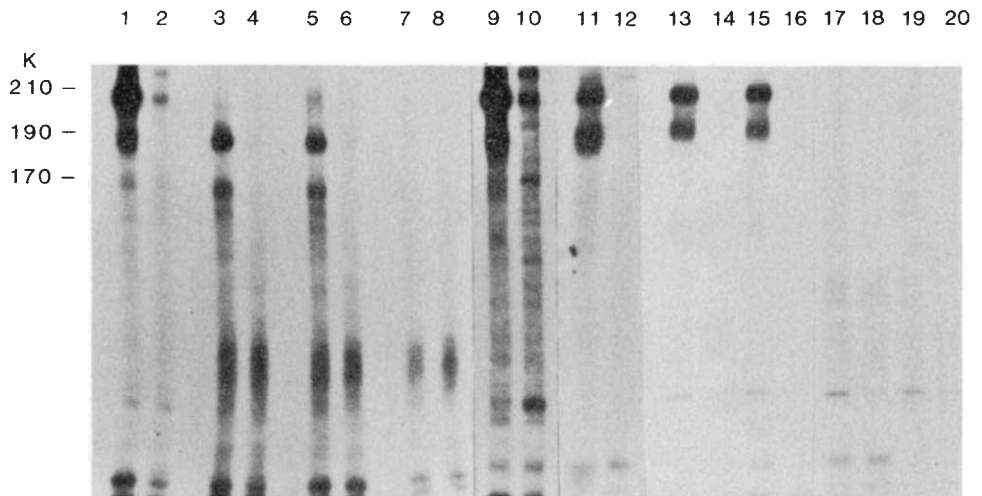
Fig. 2 Southern blot analysis of patient DNA samples and hybridization with a genomic *bcr* probe⁴. Restriction enzymes used were *Eco*RI (lanes 1-4), *Bgl*II (lanes 5-8) and *Hind*III (lanes 9-12). Lanes 1, 5, 9: normal granulocytes; lanes 2, 6, 10: Ph-positive ALL patient 1; lanes 3, 7, 11: Ph-positive ALL patient 2; lanes 4, 8, 12: Ph-positive CML patient.

Methods. DNA was prepared by cell lysis, proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation as described³⁰. Genomic DNA (20 µg) was digested with restriction endonucleases (2 units per µg DNA) in conditions recommended by the supplier (International Biotechnologies, Inc.), size fractionated by electrophoresis in a 0.8% agarose gel, denatured and transferred to nitrocellulose filters as described by Southern³¹. A 3', 1.2 kb *bcr* (*Hind*III-*Bgl*II) plasmid probe (Oncogene Science, Inc.) was used⁴. The probe was nick-translated to a specific activity of $1-2 \times 10^8$ c.p.m. per µg DNA. Hybridization was carried out overnight at 42°C in the presence of 10% dextran sulphate. The filters were then washed at 60°C for 1 h in 0.1×SSC (1×SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) containing 0.1% sodium dodecyl sulphate (SDS), air dried and autoradiographed.

Fig. 3 Immune complex kinase assays. Antisera used were anti-abl 389-403 (lanes 1, 2, 3, 4, 13, 14, 17, 18), anti-abl3 (lanes 5, 6, 9, 10) and anti-bcr3 (lanes 7, 8, 11, 12, 15, 16, 19, 20). In even lanes, the reactivity of the antisera was blocked by addition of cognate peptide. Lanes 1, 2, 9-12: K562 cells (CML cell line); lanes 3-8: Ph-positive ALL patient 2; lanes 13-16: Ph-positive ALL patient 1; lanes 17-20: Ph-negative ALL patient.

Methods. Antisera were made against peptides representing the predicted hydrophilic domain of v-abl (anti-abl 389-403)¹⁰, a predicted high term potential domain of c-abl based on the Chou-Fasman predictive model for protein secondary structure³² and a region within *bcr* exon 3⁸. Methods were as previously

described¹⁰; briefly, peptides were made using solid phase synthesis; cysteine residues were added to the carboxyl termini during synthesis for MBS cross-linking through free sulphhydryl groups to the carrier protein keyhole limpet haemocyanin and antigen was prepared by emulsifying at 1:1 ratios 0.2 mg of cross-linked peptide in Dulbecco's phosphate buffered saline with 1.5 ml of Freund's adjuvant. Subcutaneous injections of rabbits were administered at multiple sites. Three booster injections of antigen suspended in incomplete Freund's adjuvant were given at two-week intervals. Blood samples were prepared for immune complex kinase assays by treating with 5 volumes of 1.22% ammonium oxalate to lyse red blood cells and Ficoll Hypaque gradient centrifugation to enrich for immature cells. Each sample was divided into aliquots of 5×10^7 cells. Each aliquot was disrupted with 1 ml of lysis buffer (1% Triton X-100, 5 mM EDTA, 100 mM NaCl, 5 mM phenylmethylsulphonylfluoride, 100 KIU ml⁻¹ Trasylol, in 10 mM sodium phosphate, pH 7.5) with 20 strokes in a tight-fitting Wheaton homogenizer. Lysates were subjected to a 10,000g centrifugation for 10 min at 4°C. The supernatants were then incubated with 5 µl antisera for odd lanes, and a mixture of 5 µl antisera and 5 µl of 1 mg ml⁻¹ cognate peptide for even lanes, for 1 h on ice. The resulting immune complexes were then precipitated by incubation with 10 µl of Pansorbin for 20 min on ice. Immunoprecipitates were washed twice with wash buffer (0.1% Triton X-100, 150 mM NaCl, in 10 mM sodium phosphate, pH 7.5). The pellets were drained, resuspended in 50 µl of 20 mM HEPES (pH 7.0), containing 0.1% Triton X-100 and 150 mM NaCl, then reacted with 50 µl of a mixture containing 5 µCi [γ -³²P] ATP (50 Ci mmol⁻¹), 20 mM MnCl₂, 0.1% Triton X-100 in 20 mM HEPES (pH 7.0), for 10 min on ice. The reaction was terminated by addition of 3 ml of buffer containing 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate (DOC), 100 mM NaCl, 5 mM EDTA, in 10 mM sodium phosphate, pH 7.5. The immune complexes were washed twice with the above buffer and then prepared for electrophoresis according to Laemmli³³. Phosphoproteins were electrophoretically resolved according to molecular weight on a 8% sodium dodecyl sulphate polyacrylamide gel and sized with prestained markers. The gel was exposed overnight on Kodak XAR-5 film.



ferase and negative myeloperoxidase in immunocytochemical stains of bone marrow cells¹⁸. In patient 1, cytogenetic analysis revealed 45, XX, -7,t(9;22)(q34;q11) in 100% of bone marrow metaphases; in patient 2, 46,XY,t(9;22)(q34;q11) (Fig. 1).

To determine whether the 22q11 breakpoints occurred within *bcr*, we performed Southern blot analysis using a 3' 1.2-kb *Hind*III-*Bgl*II genomic *bcr* probe (Oncogene Science). Rearrangement in *bcr* in DNA derived from ALL patient 1 was observed after digestion with restriction enzymes *Eco*R1, *Bgl*II, and *Bam*HI, but not *Hind*III (Fig. 2, lanes 2, 6 and 10, and data not shown). A similar pattern of rearrangement, with localization of the breakpoint between exons 2 and 4 of *bcr*, was seen in 7 of 7 CML patients in lymphoid blast crisis (M. Shtalrid, unpublished observations) and in 8 of 8 CML patients in benign phase or myeloid blast crisis. Representative data are shown in Fig. 2, lanes 4, 8 and 12. In contrast, rearrangement in *bcr* was not detected in Ph-positive ALL patient 2 (Fig. 2, lanes 3, 7 and 11). As the *C_λ* gene resides at 22q11¹⁹, we reprobbed the filters with a genomic *C_λ* probe²⁰. Rearrangement was not observed (data not shown), indicating that the breakpoint on chromosome 22q11 in ALL patient 2 does not occur within the *bcr* or *C_λ* gene. These results are consistent with recent work by Erikson *et al.*²¹ showing a lack of *bcr* rearrangement in 3 of 5 Ph-positive ALL patients. Using *in situ* hybridization on cells obtained from one of these patients, they determined that the break at 22q11 occurred between the *C_λ* and the *bcr* gene. Therefore, the location of the breakpoint on chromosome 22 in a significant proportion of Ph-positive ALL patients may be different from that in Ph-positive CML patients, despite an identical cytogenetic marker.

As the protein products of genes are the molecules ultimately responsible for phenotype, it was of interest to examine the

c-abl protein expressed in these two different Ph-positive ALL patients. Therefore, immune complex kinase assays using synthetic peptide sera developed against the predicted hydrophilic domain of v-abl (anti-abl389-403)¹⁰, a predicted high term potential region within c-abl (anti-abl3), and a region within *bcr* exon 3 (anti-bcr3), were performed. A 210K protein, p210^{bcr-abl}, was detected in K562 cells (CML blast crisis cell line) by the two anti-abl sera and by the anti-bcr serum (Fig. 3, lanes 1, 2 and 9-12). The 190K protein in K562 cells, detected in variable amounts by these three antisera, is felt to represent a proteolytic fragment of p210^{bcr-abl} (ref. 11). The protein p210^{bcr-abl} was also present in 14 of 14 (100%) Ph-positive CML patients, three of whom were in lymphoid blast crisis, and in Ph-positive ALL patient 1 (Fig. 3, lanes 13-16 and data not shown). In contrast, p210^{bcr-abl} was not seen in Ph-positive ALL patient 2, who did not have *bcr* rearrangement. Unexpectedly, proteins of *M_r* 190K and 170K were found in this patient using both anti-abl389-403 and anti-abl3 sera (Fig. 3, lanes 3 and 5). These proteins were abl-specific as they were not detected in assays in which the reactivity of the anti-abl sera was blocked by prior addition of cognate peptide (Fig. 3, lanes 4 and 6). Furthermore, detection of p190 and p170 by two anti-abl sera, made against different domains within the abl protein, provides cogent evidence that these are bona fide c-abl proteins. The p190 observed in ALL patient 2 is distinct from the 190K proteolytic fragment seen in Ph-positive CML, since anti-bcr3 serum does not detect the former protein (Fig. 3, lane 7), but clearly reacts with the latter protein (Fig. 3, lane 11). The amount of p170 relative to p190 varied in different repeats of the experiment, suggesting that p170 may be a proteolytic fragment of p190. The protein p190 was not found in a Ph-negative ALL patient (Fig. 2, lanes 17-20), supporting a unique association between this

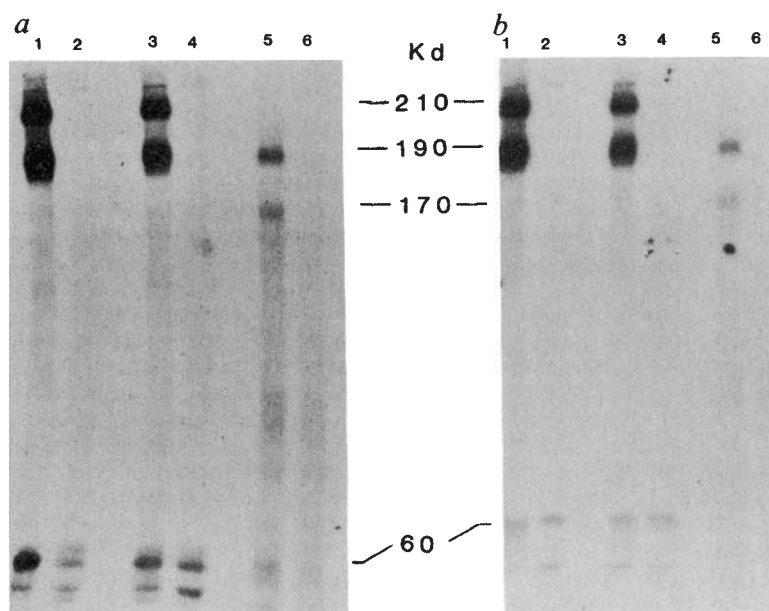


Fig. 4 Immune complex kinase assays *a*, before and *b*, after KOH hydrolysis of the gels. Antisera used were anti-*bcr3* (lanes 1, 2) and anti-*abl389-403* (lanes 3-6). In even lanes, the reactivity of the antisera was blocked by incubation with cognate peptide. Lanes 1-4, K562 cells; lanes 5 and 6, Ph-positive ALL patient 2.

Methods. Methodology for immune complex kinase assays was identical to that described for Fig. 3. Alkali treatment of gels was performed as follows²². After the dried gel was autoradiographed (*a*), it was swollen in 15 volumes of 1 M KOH and incubated at 55 °C for 2 h. The gel was then neutralized and washed with gentle shaking, with four changes of 10% acetic acid + 10% isopropanol for at least 2 h. The gel was then dried again and autoradiographed (*b*).

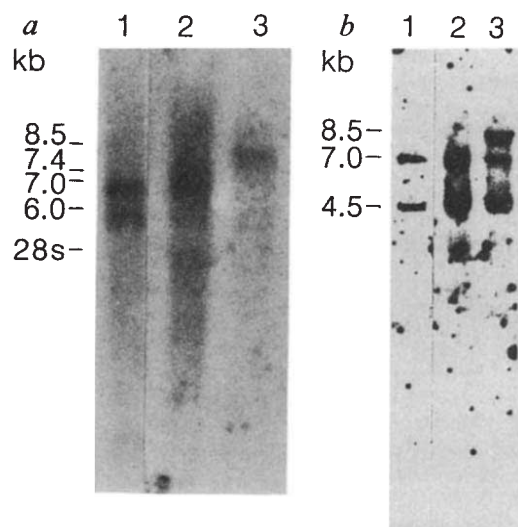


Fig. 5 Northern blot analysis of mRNA from patient samples and hybridization with a genomic *c-abl* probe²³ (*a*) and a *bcr* cDNA probe⁸ (*b*). Lane 1, Ph-negative acute promyelocytic leukaemia cell line HL-60; lane 2, Ph-positive ALL patient 2; lane 3, Ph-positive CML patient.

Methods. RNA was prepared using the guanidinium/caesium chloride method³⁴. Polyadenylated RNA was selected by chromatography on oligo (dT)-cellulose columns. The polyadenylated RNA (10 µg per lane) was size fractionated by electrophoresis in 1.1% agarose gels containing 2.2 mol l⁻¹ formaldehyde, transferred to nitrocellulose filters with 20× SSC, and hybridized to radiolabelled ³²P-DNA probes at 42 °C for 12 h by a modification of a described method³⁵. The probes were radiolabelled by nick translation to a specific activity of 1-3 × 10⁸ c.p.m. µg⁻¹ of DNA. The filters were then washed in 0.1× SSC, dried, and autoradiographed with intensifying screens for seven days at -70 °C. The *c-abl* probe was derived from a subclone of a *c-abl*-containing cosmid. We used an *EcoRI*-*Bam*HI fragment corresponding to the most 5' *v-abl* hybridizing region²³. To probe for *bcr* we used a *bcr* cDNA probe (0.45-kb *EcoRI*-*Pst*I fragment)⁸. An aberrant 7.4-kb *c-abl* transcript was detected in the Ph-positive ALL cells (*a*, lane 2). In contrast, the characteristic 8.5-kb *bcr-abl* mRNA was detected in the Ph-positive CML cells by both the *c-abl* and *bcr* probe (*a* and *b*, lane 3). Only the normal 4.5- and 6.7-kb *bcr* was present in the ALL cells (*b*, lane 2) confirming the absence of these *bcr* sequences in the aberrant 7.4-kb *c-abl* message. All lanes were run on the same gel.

protein and Ph-positive ALL; p190 and p170 remained visible after KOH hydrolysis²² of the gel consistent with phosphorylation occurring on tyrosine (Fig. 4).

Further evidence confirming the novel nature of *c-abl* expression in ALL patient 2 was obtained from Northern blot analysis (Fig. 5*a*). Hybridization with a genomic *c-abl* probe²³ revealed the characteristic 8.5-kb *bcr-abl* mRNA in a Ph-positive CML patient whereas Ph-positive ALL patient 2 expressed an aberrant 7.4-kb *c-abl* transcript. The 7.4-kb mRNA was not detected after rehybridization of the same filters with a *bcr* complementary DNA (0.45-kb *EcoRI*-*Pst*I fragment) probe⁸ (Fig. 5*b*), confirming the absence of these *bcr* sequences in this aberrant *c-abl* message. The difference in size between the aberrant transcripts found in the ALL and CML patients is consistent with production of a 190K rather than a 210K protein.

Our observations suggest that p190 is a novel aberrant *c-abl* protein product. As a similar pattern of *c-abl* expression was not observed in multiple CML patients, including those whose disease evolved to lymphoid blast crisis, p190^{*c-abl*} may be uniquely associated with development of acute lymphoid rather than chronic myeloid leukaemia. In this regard, it is of interest that *v-abl* causes transformation of lymphoid cells *in vitro*²⁴ and

murine lymphomagenesis *in vivo*²⁵. The common *abl* exon is an acceptor for splices from at least four 5' *c-abl* exons²⁶, as well as two *bcr* exons²⁷. The substitution of the normal 5' end of *abl* with *bcr* sequences is presumed to convert *c-abl* from a proto-oncogene to an oncogene, just as *gag* sequences do for *v-abl*²⁸. Therefore, the pivotal differences between *v-abl* and *bcr-abl* as opposed to *c-abl* lie in the replacement at the amino terminus. It is possible, that the amino termini of p190^{*c-abl*} and p160^{*gag-abl*} have undergone substitution with sequences that activate the tumorigenic potential of these molecules for lymphoid rather than myeloid cells. Alternatively, the rearrangement leading to p190^{*c-abl*} may be one which is favoured in lymphoid cells. In this case, the nature of the N-terminal sequences appended to the residual *c-abl* sequences may be irrelevant to its activation, with the critical event being the loss of regulatory N-terminal *c-abl* sequences.

Our study demonstrates that similar chromosomal abnormalities do not necessarily yield identical genomic events. At least two mechanisms of activation of *c-abl* exist as a result of the Ph translocation in ALL: (1) *c-abl* may be fused with the *bcr* moiety in a configuration indistinguishable from that found in Ph-positive CML; (2) *c-abl* may be fused with other, as yet

undetermined sequences. These sequences could include an upstream *bcr* fragment, or another gene, instead of *bcr*. It may be that Ph-positive ALL represents a nosologic continuum, with those patients in whom the salient molecular characteristic is formation of a *bcr-abl* protein representing the end-stage of CML which escaped detection during its early, often asymptomatic, benign phase; other patients, in whom alternate mechanisms of activation of *c-abl* occur, may be predisposed to presentation with acute lymphoid leukaemia without a preceding benign myeloid phase. Further investigation of *c-abl* expression in Ph-positive ALL is in progress and should help elucidate the role of molecular variants of this gene in acute lymphoid and chronic myeloid tumorigenesis.

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1. Nowell, P. C. & Hungerford, D. A. *Science* **132**, 1197-1200 (1960).
2. Rowley, J. D. A. *Rev. Genet.* **14**, 17-39 (1980).
3. Rowley, J. D. *Nature*, **243**, 290-293 (1973).
4. Groffen, J. *et al. Cell* **36**, 93-99 (1984).
5. Heisterkamp, N. *et al. Nature* **315**, 758-761 (1985).
6. Heisterkamp, N. *et al. Nature* **306**, 239-242 (1983).

A novel *abl* protein expressed in Philadelphia chromosome positive acute lymphoblastic leukaemia

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The Philadelphia (Ph) chromosome breakpoints in chronic myelocytic leukaemia are clustered on chromosome 22 band q11 in a 5.8-kilobase (kb) region designated *bcr*¹. The *c-abl* proto-oncogene is translocated from chromosome 9 band q34 into *bcr* and the biochemical consequence of this molecular rearrangement is the production of an abnormal fusion protein *bcr-abl* p210 with enhanced protein-tyrosine kinase activity^{2,3} compared to the normal p145 *c-abl* protein. The Ph chromosome translocation is also seen in some acute lymphoblastic leukaemias with B-cell precursor phenotypes^{4,5} some of which have *bcr* rearrangement (*bcr*⁺) and some do not (*bcr*⁻)^{6,7}. We present evidence that the Ph⁺, *bcr*⁻ leukaemias are associated with a novel p190 *abl* kinase. We propose that acute lymphoblastic leukaemias that are *bcr*⁺, p210⁺ are probably lymphoid blast crises following a clinically silent chronic phase of chronic myelocytic leukaemia arising in multipotential stem cells whereas *bcr*⁻, p190⁺ cases are *de novo* acute lymphoblastic leukaemias arising in more restricted precursors.

We obtained four cases of Ph⁺ acute lymphoblastic leukaemia leukaemia (ALL) for our study (F.B., F.Y., P.N. and A.E.). The

7. Bartram, C. R. *et al. Nature* **306**, 277-280 (1983).
8. Shtivelman, E., Lifshitz, B., Gale, R. P. & Canaani, E. *Nature* **315**, 550-554 (1985).
9. Stam, K. Jr *et al. New Engl. J. Med.* **313**, 23-27 (1985).
10. Klotzner, W. *et al. Virology* **140**, 230-238 (1985).
11. Konopka, J. B., Watanabe, S. M. & Witte, O. N. *Cell* **37**, 1035-1042 (1984).
12. Konopka, J. B. & Witte, O. N. *Molec. cell. Biol.* **5**, 3116-3123 (1985).
13. Ben-Neriah, Y., Daley, G. Q., Mes-Masson, A., Witte, O. N. & Baltimore, D. *Science* **233**, 212-214 (1986).
14. Third International Workshop on Chromosomes in Leukemia, *Cancer Genet. Cytogenet.* **4**, 111-137 (1980-81).
15. Catovsky, D. *et al. Am. J. clin. Path.* **72**, 736-745 (1979).
16. Catovsky, D. *Br. J. Haemat.* **42**, 493-498 (1979).
17. Koeffler, H. P. & Golde, D. *New Engl. J. Med.* **304**, 1201-1209 (1981).
18. Bollum, F. J. *Blood* **54**, 1203-1206 (1979).
19. Erikson, J., Martinis, J. & Croce, C. M. *Nature* **294**, 173-175 (1981).
20. Hieter, P. A., Hollis, G. F., Korsemeyer, S. J., Waldmann, T. A. & Leder, P. *Nature* **294**, 536-540 (1981).
21. Erikson, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 1807-1811 (1986).
22. Cooper, J. A. & Hunter, T. *Molec. cell. Biol.* **1**, 165-170 (1981).
23. De Klein, A. *et al. Nature* **300**, 765-767 (1982).
24. Rosenberg, N. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 1932-1936 (1978).
25. Abelson, H. T. & Rabstein, L. S. *Cancer Res.* **30**, 2213-22 (1970).
26. Ben-Neriah, Y., Bernards, A., Paskind, M., Daley, G. Q., & Baltimore, D. *Cell* **44**, 577-585 (1986).
27. Heisterkamp, N. *et al. Nature* **315**, 758-765 (1985).
28. Konopka, J. B. & Witte, O. N. *Biochim. biophys. Acta* **823**, 1-18 (1985).
29. Trujillo, J. M., Cork, A., Ahearn, M. J., Youness, E. L. & McCredie, K. B. *Blood* **53**, 695-706 (1979).
30. Wong-Staal, F., Reitz, M. S. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2032-36 (1979).
31. Southern, E. M. *J. molec. Biol.* **98**, 503-507 (1975).
32. Chou, P. & Fasman, G. D. *Adv. Enzym.* **47**, 45-147 (1978).
33. Laemmli, U. *Nature* **227**, 68-72 (1970).
34. Chergwin, J. M., Przybyla, A. E., MacDonald, R. J., Rutter, W. J. *Biochemistry*, **18**, 5294-5298 (1979).
35. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5206 (1980).

Table 1 Summary data of Ph⁺ acute lymphoblastic leukaemia cases

Patient	Age/sex	Ph chromosome	<i>bcr</i>	<i>abl</i> protein
F.B.	54/F	+	Rearranged	p210
F.Y.	35/F	+	Normal	p190
P.N.	46/M	+	Normal	p190
A.E.	14/M	+	Normal	p190
(9; 17; 22)*				

* Banding was performed on case A.E., which showed the same chromosomal breakpoints on chromosome 9 and 22 (bands 9q34 and 22q11) as in Ph⁺ CML, although the cells were unusual in having a complex variant translocation involving chromosomes 9, 17 and 22.

Ph chromosome was observed by cytogenetic analysis in each case. Immunophenotyping of the blast cells revealed a common ALL phenotype⁸ in three patients (F.B., F.Y., P.N.) and an unclassifiable phenotype in patient A.E. (co-expression of lymphoid and myeloid antigens).

The *bcr* status in the leukaemic DNA was determined using a large *bcr* probe (Fig. 1a) and four different digests, *Bgl*II, *Bam*HI, *Hind*III and *Eco*RI. Rearrangement of *bcr* was observed in the DNA from patient F.B. while only the normal restriction enzyme fragments were present in DNA from patients F.Y., P.N. and A.E. (Fig. 1b, and data not shown). These results confirm earlier reports that Ph⁺ ALL is heterogenous with respect to involvement of the *bcr*^{6,7}, in contrast to Ph⁺ chronic myelocytic leukaemia (CML), where *bcr* rearrangement has been observed in all 40 cases we have examined (data not shown).

As the 5.8-kb *bcr* is not involved in 3 out of the 4 Ph⁺ ALL, it was important to investigate whether *abl* was affected by the translocation event. Using an *abl*-specific affinity purified polyclonal antiserum, *abl*-related proteins were immunoprecipitated from leukaemic cells of the four Ph⁺ ALL cases. The *in vitro* phosphorylated immunoprecipitates from cells of patient F.B. (Ph⁺ *bcr*⁺) or Nalm-1 (a cell line derived from a CML lymphoid blast crisis⁹ which is also Ph⁺, *bcr*⁺) revealed a phosphoprotein of relative molecular mass 210,000 (*M*_r 210K) called p210, which is the appropriate size for the *bcr-abl* fusion protein (Fig. 2a). In contrast, a phosphoprotein of *M*_r 190 K (p190) was seen in each of the three Ph⁺, *bcr*⁻ ALL patients (Fig. 2a). In control experiments, neither p210 nor p190 was observed in two Ph⁻,

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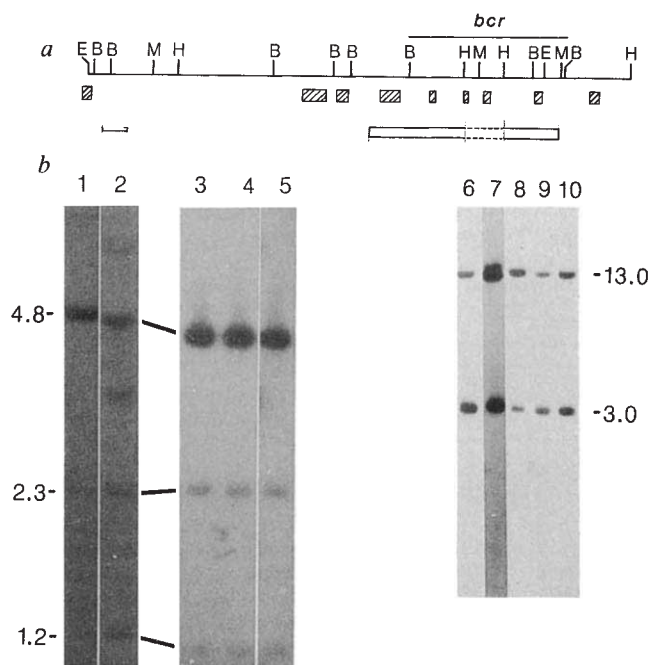


Fig. 1 DNA analysis to detect *bcr* rearrangements. *a*, Restriction map of the characterised *bcr* of chromosome 22 band q11. Hatched boxes, known positions of exons; open box, *bcr* probe (the dotted region was deleted because it contains repetitive sequences). The line above the map denotes the region designated *bcr*. Restriction enzyme sites shown thus: E, *EcoRI*; B, *BglII*; M, *BamHI*; H, *HindIII*. Scale bar, 1 kb. *b*, DNA was digested with *BglII* (lanes 1–5) or *BamHI* (lanes 6–10), electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose¹⁶ and allowed to hybridize to the *bcr* probe depicted in *a*. DNAs were isolated from (lanes 1 and 6) control cells of Bri-7, an EBV transformed cell line without the Ph chromosome; lanes 2 and 7, cells from patient F.B.; lanes 3 and 8, cells from patient F.Y.; lanes 4 and 9, cells from patient P.N.; and lanes 5 and 10, cells from patient A.E. Sizes of unrearranged bands in kilobases.

bcr⁺ ALL cases or in three other Ph⁺ cell lines—HPBALL (T cell; Fig. 2a); KG1 (myeloid cell) and Nalm-6 (B cell) (data not shown). To exclude the possibility that specific degradation of the p210 to p190¹⁰ occurs in these samples during cell lysis, the *in vitro* kinase assay was repeated using extracts obtained from a mixture of cells from patients F.B. (p210) and A.E. (p190) (Fig. 2b). Both p210 and p190 were clearly visible, showing that the novel p190 *abl* protein is unlikely to be a degradation product of p210. In addition, protein extracts from the leukaemia cells from six patients diagnosed as CML in lymphoid blast crisis contained the p210 species (data not shown). If a specific protease were associated with lymphoid blast cells, we would expect a similar observation in this series of samples.

Previously, *in vitro* phosphorylation of p210 has been shown to occur primarily on tyrosine with a trace amount of phosphoserine¹⁰. Phosphoaminoacid analysis¹¹ of *in vitro* phosphorylated p190 demonstrated that, as for p210, >95% of the ³²P-incorporation occurred on tyrosine residues (data not shown). The p190 appears to have enhanced protein-tyrosine kinase (PTK) activity similar to that of the p210 but because of the way we measure the *abl* protein in an *in vitro* assay we cannot exclude the possibility that the increase we observe in p190 PTK level in the cell might be due to an increase in the *abl* protein level.

We looked for rearrangements in the DNA upstream of the *c-abl* gene. When the DNA blots used for *bcr* analysis were re-hybridized with the 520-bp *EcoRI* fragment localised 17 kb upstream of the *v-abl* homologous coding sequences (designated 0.52E, see ref. 12) (Fig. 3a) an additional band was noted in both the *BglII* and *BamHI* digests of patient F.Y. (Fig. 3b). It

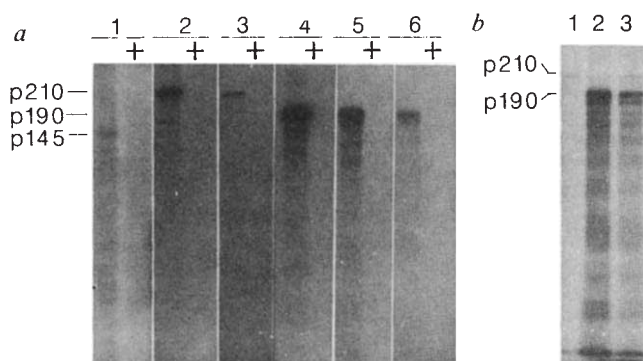


Fig. 2 *In vitro* protein tyrosine kinase assay of *abl* related proteins. *a*, Proteins were immunoprecipitated in the absence of presence (+) of the cognate peptide 4. This peptide sequence is just 3' to the *abl* protein kinase domain¹⁷. Protein preparations were isolated from: lane 1, HPB ALL; lane 2, Nalm-1; lane 3, patient F.B.; lane 4, patient F.Y.; lane 5, patient P.N.; lane 6, patient A.E. *b*, Cells from F.B. (lane 1) and A.E. (lane 2) were mixed (lane 3) before kinase analysis. The autophosphorylated proteins were detected by autoradiography.

Methods. Mononuclear cells from clinical samples were isolated on Ficoll-Hypaque (Pharmacia) gradients. Cells (~10⁷) were lysed in 1 ml of buffer containing 1% Triton X-100, 0.05% SDS, 10 mM sodium phosphate pH 7.0, 150 mM NaCl, 5 mM phenylmethylsulphonyl fluoride, 70 µg ml⁻¹ of each of the following proteinase inhibitors: α₂-macroglobulin, bestatin, aprotinin, trypsin inhibitor and leupeptin. The samples were centrifuged for 10 min at 16,000g to remove insoluble material and divided into two tubes containing affinity-purified antibody which we have raised against peptide 4 and 30 µl of packed resin bound α₂-macroglobulin (Boehringer-Mannheim). To one tube (+) 0.5 mM of the cognate peptide was added. After 15 min incubation on ice the tubes were again centrifuged and the supernatant transferred to a tube containing 10 µl packed protein A-Sepharose beads. The tubes were mixed gently and allowed to incubate for 20 min on ice. The beads were washed with lysis buffer, without SDS, resuspended in 20 µl of 20 mM PIPES pH 7.0, 20 mM MnCl₂, and 25 µCi of >5000 Ci mmol⁻¹ [³²P]ATP (Amersham) and incubated for 5 min at 30 °C (ref. 2). The beads were then washed twice with lysis buffer, boiled in 100 µl sample buffer, and electrophoresed through an 8% polyacrylamide gel¹⁸. The fixed gel was dried and autoradiographed for several hours (lanes 2–6) or overnight (lane 1) at -70 °C using Kodak XAE-5 film.

is not surprising that rearrangements were not observed in DNA from the other patients, since Ph⁺ CML, the break on chromosome 9 band q34 can occur in a region 10 kb to >100 kb upstream¹³ of the *v-abl* homologous region of the *c-abl* gene.

The mechanism of activation of *c-abl* p190 PTK in Ph⁺, *bcr*⁺ ALL is unclear. We isolated poly(A)⁺RNA from frozen leukaemic cells from patient A.E. and compared it to poly(A)⁺ RNA from KG1 and K562 cell lines on RNA blots. KG1, a myeloid cell line, expresses the normal p145 *abl* protein (data not shown) as well as the normal 6.0 and 7.0 kb *abl* mRNA species (Fig. 4, lane 3). K562 is a cell line derived from a patient diagnosed as CML in blast crisis; it contains the p210¹⁰ and expresses the normal *abl* as well as the 8.7 kb *bcr-abl* hybrid mRNAs (ref. 14 and Fig. 4, lane 1). No abnormal sized transcripts were observed in the RNA from A.E. (Fig. 4, lane 2) although there appears to be more of the 7.0-kb compared to the 6.0-kb mRNA species. In addition, abnormal *c-abl* transcripts have not been detected in two other such cases studied^{6,7}, but the predicted size of the hybrid *c-abl* transcript could be very similar to the normal *c-abl* mRNA and therefore difficult to resolve by gel electrophoresis.

Our observations suggest that two different molecular mechanisms involving *c-abl* on chromosome 9 and genetic information on chromosome 22 band q11 can generate enhanced kinase activity or result in stabilization or elevated expression of the *c-abl* protein in Ph⁺ ALL. One involves the *bcr* identified

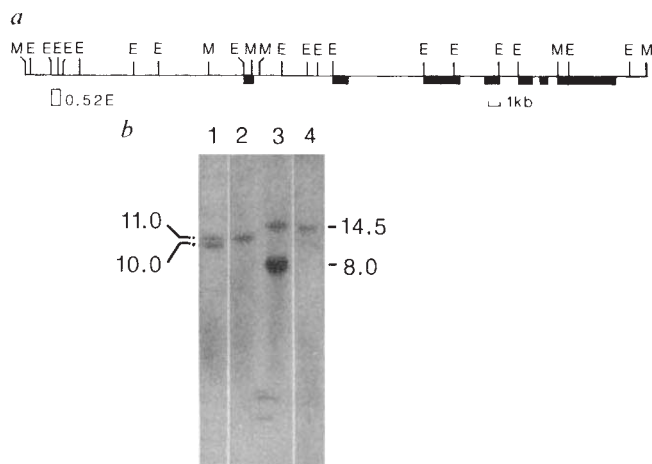


Fig. 3 DNA analysis to detect *abl* rearrangements. *a*, Restriction map for the 5' region of *c-abl* located on chromosome 9 band q34. Closed boxes, regions homologous to *v-abl*; open box, the probe 0.52E. Abbreviations for enzyme sites are as in Fig. 1*a*. Scale bar, 1 kb. *b*, DNA from F.Y. (lane 1 and 2) or Bri-7 (lanes 3 and 4) was digested with *Bgl*II (lanes 1 and 2) or *Bam*HI (lanes 3 and 4) and treated as in Fig. 1*b*. The blot was hybridized to the upstream *abl* probe labelled by the random primer method¹⁹. Sizes of fragments hybridizing in kb.

originally in Ph^+ CML while the other appears to involve sequences outside the designated 5.8-kb *bcr*. Because the latter mechanism has not been detected in a large series of chronic phase Ph^+ leukaemia (CML) cases, there appears to be some association between these two mechanisms and disease status. Note that we have not determined whether another region of the *bcr* (or *phl*) gene on chromosome 22, might be involved in this translocation. Interestingly, we have recently screened 13 cases of ALL without the Ph chromosome and identified one which appears to express a normal (p145) sized *abl* related protein with enhanced PTK activity or elevated level of the protein (C. Girdham and K.K.K., data not shown). This may represent a third mechanism for the activation of the *c-abl* proto-oncogene.

It has been suggested that Ph^+ , bcr^+ ALL, which produces an 8.7-kb aberrant *bcr-abl* transcript can be considered to be Ph^+ CML in which lymphoid blast crisis has occurred after an undetected chronic phase arising in multipotential progenitor cells⁶ (see also refs 4, 5). Our DNA data on patient F.B. which shows rearrangement in the *bcr* and the cellular expression of p210 is consistent with this hypothesis. Ph^+ , bcr^- ALL might then represent *de novo* B lymphocyte precursor ALL. It is uncertain at present, owing to the small number of cases in our study, whether Ph^+ , bcr^- ALLs which produce the novel *c-abl*

p190 protein are indeed clinically distinct from Ph^+ , bcr^+ ALL. This can perhaps be assessed by testing for *c-abl* p190 PTK in remission and by clinical follow up of remission duration and disease evolution of relapse in a larger series of patients. What is clear is that this group includes adults as well as children, in contrast to previous studies which suggested that Ph^+ , bcr^- ALL is primarily a childhood disorder^{7,15}. A summary of the Ph^+ ALL cases in our study is shown in Table 1.

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- Groffen, J. *et al. Cell* **36**, 93-99 (1984).
- Konopka, J. B. & Witte, O. N. *Molec. cell. Biol.* **5**, 3116-3123 (1985).
- Ben-Neriah, Y., Daley, G. Q., Mes-Masson, A.-M., Witte, O. N. & Baltimore, D. *Science* **233**, 212-214 (1986).
- Greaves, M. F. *Chronic Granulocytic Leukaemia* (ed. Shaw, M. T.) 15-47 (Praeger, New York, 1982).
- Catovsky, D. *Br. J. Haemat.* **42**, 493-498 (1979).
- De Klein, A. *et al. Blood* **6**, 1369-1375 (1986).
- Erikson, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 1807-1811 (1986).
- Greaves, M. F., Janossy, G., Peto, J. & Kay, H. *Br. J. Haemat.* **48**, 179-197 (1981).
- Minowada, J., Koshida, H., Janossy, G., Greaves, M. F. & Bollum, F. J. *Leuk. Res.* **3**, 261-266 (1979).
- Konopka, J. B., Watanabe, S. M. & Witte, O. N. *Cell* **37**, 1035-1042 (1984).
- Heath, J. K., Mahadevan, L. & Foulkes, J. G. *EMBO J.* **5**, 1809-1814 (1986).
- Heisterkamp, N. *et al. Nature* **306**, 239-242 (1983).
- Grosveld, G. *et al. Molec. cell. Biol.* **6**, 607-616 (1986).
- Shtivelman, E., Lifshitz, B., Gale, R. P. & Canaani, E. *Nature* **315**, 550-554 (1985).
- Rodenhuis, S., Smets, L. A., Slater, R. M., Behrendt, H. & Veerman, A. *New Engl. J. Med.* **313**, 51 (1985).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Konopka, J. B. *et al. J. Virol.* **51**, 223-232 (1984).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).
- Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **137**, 266-267 (1984).
- Wiedemann, L. M., Burns, J. H. & Birnie, G. D. *EMBO J.* **2**, 9-13 (1983).
- Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408-1412 (1972).
- Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, T. *Biochemistry* **16**, 4743-4751 (1977).

Rapid rotation of flagellar bundles in swimming bacteria

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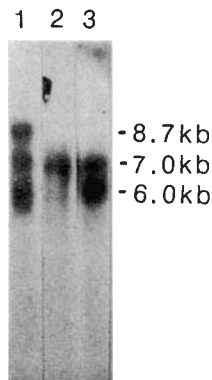
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A bacterial flagellum is driven by a reversible rotary motor¹⁻³. The power input is determined by protonmotive force and proton flux, the power output by torque and speed: interrelationships between these parameters provide important clues to motor mechanisms. Here we describe the relationship between torque and speed at constant protonmotive force. The measurements are analogous to those that could be made by plugging an electric motor into a constant-voltage outlet, varying the external load, and determining the torque delivered at different speeds. We used suspensions of metabolizing cells of a motile *Streptococcus*, varied the external load by changing the viscosity of the medium, determined motor speed from the frequency of vibration of the cell body, and inferred motor torque from the rate of body rotation. The flagellar bundles rotate more rapidly than formerly supposed, at rates that increase linearly with temperature. The torque delivered by the flagellar motor drops linearly with speed. At high speed, the torque-generating cycle associated with the transfer of one proton appears to dissipate free energy in a series of small steps.

The helical bundle of a swimming bacterium generates thrust, and the cell translates at a velocity at which this force is balanced

Fig. 4 RNA analysis for altered *abl* mRNA sequences. Poly(A)⁺ RNAs (6-10 µg) were analysed by electrophoresis, blotting and hybridized to a *v-abl* kinase domain probe. RNAs were purified from K562, a bcr^+ cell line derived from a patient with CML in blast crisis (lane 1); cells from patient A.E. (lane 2); and KGL, a myeloid cell line with no Ph involvement (lane 3). RNA sizes in kb as determined from size markers.

Methods. RNA was isolated from cells using the guanidinium thiocyanate method followed by centrifugation through CsCl^{20} and enriched for poly(A)⁺ RNA by oligo-dT chromatography²¹. The RNA was denatured, electrophoresed through a 6% formaldehyde, 0.8% agarose gel²², transferred to Genescreen Plus (NEN) and immobilized by exposure to a UV light source for 5 min followed by baking for 2 h at 80 °C. The blot was then hybridized to a nick-translated *v-abl* probe¹⁴ and autoradiographed.



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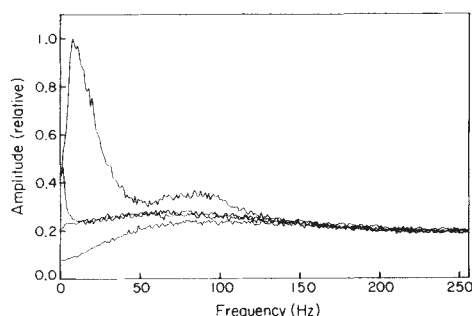


Fig. 1 Amplitude spectra of swimming cells of *Streptococcus* (top curve), of de-energized cells moving at the speed of the swimming cells (second curve), of de-energized cells undergoing Brownian movement (third curve), and of the background illumination at the same total light intensity (bottom curve).

Methods. Cells of a smooth-swimming mutant, SM197, were grown at 35 °C in KTY medium², collected at late exponential phase, washed twice by centrifugation (480g, 6 min) in an equal volume of 0.1 M sodium phosphate (pH 7.5), 0.2 M KCl, 0.1 mM EDTA, 0.01 M D-glucose, and then resuspended in this medium at a density of $\sim 4 \times 10^8$ cells ml⁻¹. An aliquot of this suspension was drawn into a flow chamber²² (0.4 mm deep) and examined at 22 °C under an inverse phase contrast microscope (see below), yielding the spectrum shown in the top curve (mean swimming speed $\pm$ s.e.m., 15.7 ± 1.1 μ m s⁻¹ for 17 cells, as judged from slow-speed playback of a video recording²³). Another aliquot was de-energized by the addition of the uncoupler FCCP (trifluoromethoxycarbonyl-cyanide phenylhydrazide, 10 μ g ml⁻¹) and either drawn through the chamber at a speed of 14.4 μ m s⁻¹ (measured at the middle of the chamber), yielding the spectrum shown in the second curve, or examined when stationary, yielding the spectrum shown in the third curve. The microscope (Nikon S-Ke) was equipped with a 100 W tungsten-halogen lamp (type FCR, driven by a constant-voltage d.c. power supply), a heat-transmitting mirror (Optical Industries 03MCS007), a CF BM $\times 40$ objective, a $\times 20$ eyepiece, a $\frac{1}{2} \times$ PFM photomicrographic attachment, and a photomultiplier tube (RCA 4886, run at 300 V). The focal plane was located midway between the top and bottom windows of the flow chamber to minimize wall effects. The diameter of the photocathode (1.8 cm), referred to the focal plane, was ~ 39 μ m. The output of the photomultiplier was passed through a current-to-voltage converter (10^7 Ω feedback), a difference amplifier (to remove the d.c. offset and amplify the a.c. signal), a double-pole low-pass filter, and a single-pole high-pass filter. The 3 dB cutoff points of these filters were set at 200 and 72 Hz, respectively. A 12-bit analogue-to-digital converter sampled data at 512 points s⁻¹ for one-second intervals and a PDP-11/34 computer computed and averaged the corresponding power spectra (400 times for the data shown). The signals arose from lateral motion of images on the photocathode, which was exposed to light from only a small region of the flow chamber, and which when scanned with the image of a cell that was stuck to glass, was found to have an output (signal less background) that rose and fell 3 or 4 times across a diameter, varying in amplitude by as much as 50%. Contributions from out-of-focus components of the images were significant, as useful spectra could be obtained on moving the plane of focus beyond the limits of the flow chamber. The basic features of the spectra, including sidebands due to non-linearities encountered with larger displacements (see text), could be reproduced by mounting the detector on the tracking microscope²⁴, suspending de-energized cells in the tracking chamber, and moving it sinusoidally with the tracker drive. Much larger signals were obtained on moving the tracking chamber sideways than up and down. De-energized cells and monodisperse polystyrene latex spheres (1.3 μ m diameter) gave identical spectra.

by viscous drag on the body; the bundle also generates torque, and the body counter-rotates (or rolls). If the longitudinal axes of the bundle and the cell body are not collinear, then the image of the body wobbles from side to side. If there is a net imbalance in the forces perpendicular to the axis of the helix, for example, if the bundle does not contain an integral number of wavelengths⁴, then the cell body also vibrates (or gyrates) at the rotation frequency of the bundle. The presence of such motion

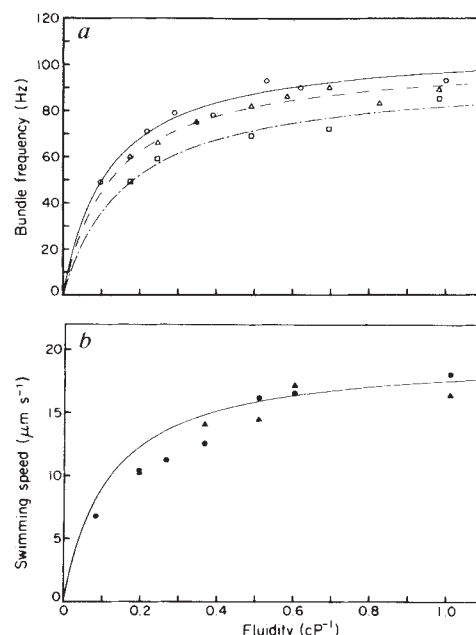


Fig. 2 Bundle frequencies (a) and swimming speeds (b) of *Streptococcus* as a function of the fluidity of the medium. The different symbols refer to cells from different cultures. The values shown at each point are means for the cell population determined from the power spectrum or the tracking data, respectively. In either case, the spread (s.d. divided by the mean) was about 25%. The fits to the data in a are shown in Fig. 3. The solid curve in b is the solid curve in a, redrawn and scaled to match approximately the swimming speeds at high fluidity.

Methods. To measure bundle frequencies, cells of strain SM197 were grown, collected, washed as described in Fig. 1. Then (open circles) cells were resuspended at 80 times the density at collection. An aliquot was mixed with 9 vols of Ficoll 400 solution of known concentration. Alternatively (open triangles and open squares), cells were pelleted once, as before, and resuspended in a solution of Ficoll in KTY medium. All spectra were taken as described in Fig. 1, except in a shallower chamber (0.15 mm deep)²³. Ficoll standards were prepared by serial dilution of a 20% w/v stock prepared in the medium in which the cells were suspended. The viscosities of these solutions were measured at 22 °C in a Cannon-Ubbelohde viscometer (viscometer constant 8.13×10^5 cm² s⁻²). The same stock was used to prepare all the Ficoll solutions. The position of the centre of the high-frequency peak was found as follows. The power spectrum for non-motile cells was smoothed with a cubic spline procedure²⁵ and subtracted from the power spectrum for the swimming cells. The difference spectrum was smoothed in the same way, and its low-frequency peak was fitted by a curve generated by computer simulation (see text). This curve was subtracted out, and the centre of the remaining (high-frequency) peak was found by eye. To measure swimming speeds, cells of the wild-type strain V4051 (ref. 26) were grown, collected and washed as described in Fig. 1, and then resuspended at 2.5 times the density at collection. An aliquot was mixed with 50 volumes of a solution of Ficoll of known concentration, and the swimming speeds of 20–30 cells were found by tracking²⁴. The following data (mean $\pm$ s.d., unless otherwise noted) were obtained for a population of 60 bacteria in the absence of Ficoll (compare ref. 27, Table 1): speed 16.8 ± 3.7 μ m s⁻¹ (mean $\pm$ s.e.m.), tumble length 0.18 ± 0.07 s, run length 1.71 ± 0.90 s, change in direction from run to run $63 \pm 14^\circ$, and change in direction during runs $26 \pm 8^\circ$. The run-tumble statistics also were Poisson.

was postulated long ago by Reichert⁵, who referred to it as *die Trichterbewegung*, or funnel movement⁶.

We measured these frequencies by projecting the images of swimming cells onto the photocathode of a photomultiplier tube whose sensitivity is spatially inhomogeneous. The output was passed through a bandpass filter and the power spectral density was computed with the fast Fourier transform. The spectra of many successive one-second data blocks were averaged. The

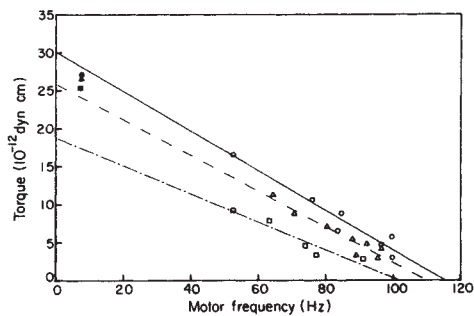


Fig. 3 Torque of the flagellar motor of *Streptococcus* as a function of speed. The open symbols are values derived from the data of Fig. 2a. The lines are least-squares fits to these data. The closed symbols are values derived from experiments on cells tethered by a flagellar filament¹¹.

Methods. For swimming cells, the torque exerted by the bundle on the cell body is given by the product of the body-roll frequency, the viscosity of the medium, and a geometrical factor that depends on the shape of the cell and its axis of rotation. We approximated the cell body as a cylinder of length $3.27 \mu\text{m}$ and width $1.27 \mu\text{m}$ rolling about an axis that intersects the cylinder axis $1.09 \mu\text{m}$ from one end of the cell at an angle of 22° . Cell sizes were determined from photomicrographs of cells that had settled on a glass surface, whereas other parameters related to the geometry were estimated by inspecting the trajectory of several cells on the video record. The corresponding drag coefficient was computed from published formulae^{28,29}. No correction was made for the additional viscous drag on the body due to the counter-rotation of the flagellar bundle or for loss of torque due to interactions of flagella within the bundle; the latter assumption is justified by the observation that free hooks² or flagellar stubs⁷ spin at rates comparable to those of flagellar bundles. We visualized the flagellar filaments with a modified Ryu stain³⁰ and found an average of 3.5 ± 0.2 flagella per cell, all of which were assumed to join in a single bundle. For tethered cells, cells from three cultures were prepared as described in Fig. 1 and tethered to a silanized cover slip¹¹. We approximated the cell body as a cylinder of length $3.04 \mu\text{m}$ and width $1.36 \mu\text{m}$ spinning about an axis perpendicular to the cylinder axis $0.32 \mu\text{m}$ away from the centre of the cell. No correction was made for the additional viscous drag due to the proximity of the cover slip³¹.

top curve of Fig. 1 shows the amplitude of this spectrum (the square-root of the power spectrum) obtained from a field of smooth-swimming cells of *Streptococcus*; the low-frequency peak is due to the roll of the cell body and the high-frequency peak to its funnel movement. The other spectra in Fig. 1 characterize noise arising from changes in the number of cells imaged on the detector, Brownian movement and photoelectron statistics.

To interpret these spectra better, we simulated the measurements by computer, moving a vibrating spot across a detector with a radial gaussian sensitivity and filtering the output. We concluded from this analysis that the low-frequency peak (Fig. 1) is skewed toward high frequencies because of harmonics of the body-roll frequency that arise from the nonlinear relation between displacement and detector output at large wobble amplitudes. The high-frequency peak, on the other hand, although broadened somewhat by sidebands generated by mixing with the low-frequency signal, remains centred at the mean bundle frequency, provided (as in Fig. 1) that the two peaks do not considerably overlap. Both peaks are broadened and smoothed by variations over the cell population⁷.

The correct body-roll frequency corresponding to each amplitude spectrum was determined by examining video recordings made of the same cell suspensions. An average was computed from the wobble frequencies of all the cells in a video field, as measured by eye and stopwatch during slow-speed playback. For the culture used in the experiments of Fig. 1, the body-roll frequency was $6.7 \pm 2.4 \text{ Hz}$ (mean $\pm$ s.d. for 20 cells). Given a bundle frequency of about 95 Hz (Fig. 1), this yields a value for

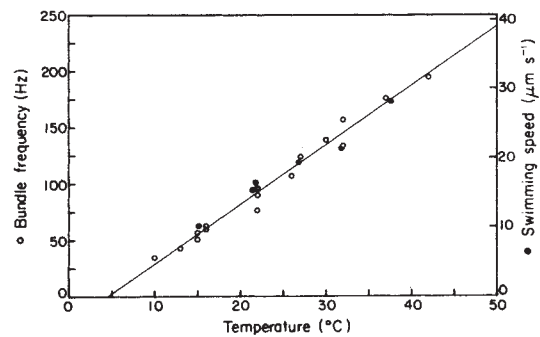


Fig. 4 Bundle frequencies ($\circ$) and swimming speeds ($\bullet$) of *Streptococcus* as a function of temperature. The values shown are the means for the cell population, determined at each point from the power spectrum or the tracking data, respectively. In either case, the spread was about 25%. The temperature was controlled as described previously¹⁰. Other conditions were as in Fig. 2 (with cells prepared in phosphate buffer). The line is a least-squares fit to the bundle-frequency data. The swimming-speed scale was adjusted so that the swimming-speed data fell along the same line.

the speed of the flagellar motor relative to the cell body of about 100 Hz.

Figure 2 shows the dependence of bundle frequency and swimming speed on the fluidity of the medium, adjusted by addition of Ficoll, a highly branched viscous agent whose solutions show newtonian properties⁸. A tenfold increase in viscosity was required to halve the bundle frequency. Swimming speeds were closely correlated with bundle frequencies, but they were slightly lower at higher viscosities: the ratio of swimming speed to bundle frequency decreased from $0.18 \pm 0.01 \mu\text{m}$ to $0.15 \pm 0.01 \mu\text{m}$ for the data shown. A possible explanation for this trend is that the bundle winds up slightly as the torque increases, decreasing its propulsive efficiency. We also measured bundle frequencies of *Streptococcus* as a function of fluidity using methylcellulose and found that the frequencies decreased monotonically with increasing viscosity (data not shown). Thus, increases in swimming speed observed on the addition of small amounts of this agent⁹ must arise from enhancement of the efficiency of flagellar propulsion, as argued previously⁸, not from changes in the speed of the flagellar motors.

Figure 3 shows the torque generated by the flagellar motor as a function of its rotation rate. The open symbols are a different representation of the data of Fig. 2a: their position on the abscissa is the sum of the body-roll and bundle frequencies, equal to the motor rotation frequency, whereas their position on the ordinate is proportional to the product of the body-roll frequency and the viscosity of the medium. The proportionality constant is determined by a geometrical drag factor and the number of filaments per bundle. The same constant was assumed for all the experiments on swimming cells. For the three cultures, the torque appears to drop linearly with increasing speed, although the relationship varies somewhat from culture to culture. The closed symbols represent the torque for tethered cells, calculated in the same fashion, but with a different geometrical drag factor. These torques correspond well to the linear extrapolation of the data for swimming cells. Note that the ratio of the two drag factors, and thus the position on the ordinate of the closed symbols relative to the open symbols, is subject to considerable uncertainty, probably up to 40%. The implications of Fig. 3 for the mechanism of torque generation are discussed below.

Figure 4 shows the dependence of bundle frequency (open symbols) and swimming speed (closed symbols) of *Streptococcus* on temperature. Both increase linearly over the range 10 to 40°C . To check for a possible temperature dependence of the protonmotive force, we tethered cells from the culture used for

the bundle-frequency measurements. Their torques were compared at 9.3, 16.0, 22.0, 27.8 and 31.6 °C and found to fall in the ratios 0.7, 0.95, 1.00, 1.02 and 1.04, respectively (torques for each of 14 cells were scaled to the torque at 22 °C and then averaged; the standard deviation for the population was ~0.05; the mean speed at 22 °C was 6.3 Hz). Artificially energized cells generate a torque that is essentially independent of temperature over the range 4–38 °C¹⁰. Assuming a linear dependence of torque on protonmotive force^{10,11}, we conclude that the protonmotive force of metabolizing cells increases by at most 10% over the temperature range 16–32 °C. Over this range, the bundle frequencies of the swimming cells increased by a factor of 2.4 (Fig. 4). A linear dependence of swimming speed on temperature has been observed for *Escherichia coli* using number-fluctuation spectroscopy¹² and cinematography¹³. Similar results were obtained for *Salmonella typhimurium* by recording motility tracks¹⁴.

We also measured the bundle frequencies and swimming speeds of *E. coli*. The mean bundle frequency of a smooth-swimming strain (HCB437) grown at 35 °C on glycerol in a minimal salts medium, collected at mid-exponential phase, and then studied in motility medium¹⁵ at 22 °C—these were the conditions used for the measurements of rotational frequencies of hooks described earlier (mean $\pm$ s.d. for 20 cells 104 ± 29 Hz)²—gave a result in the same range, 113 Hz. The mean bundle frequency for these cells was higher at 32 °C, 156 Hz. We also determined mean bundle frequencies for cells grown at 35 °C in tryptone broth and collected at mid-exponential phase. These frequencies were higher still, 191 Hz at 22 °C, and 268 Hz at 32 °C. All these measurements were made near the open edge of the chamber⁸ to ensure that the cells were well oxygenated. Cells of wild-type strain AW405 (ref. 16) were grown on tryptone broth and tracked at 32 °C in motility medium containing varying amounts of Ficoll. The dependence of swimming speed on fluidity was essentially identical to that observed for *Streptococcus* (Fig. 2b), except that the swimming speeds were higher (mean $\pm$ s.e.m. for 79 cells at fluidity 1.25 cP^{-1} , $36.4 \pm 1.0 \mu\text{m s}^{-1}$).

What can these measurements tell us about the physics of flagellar propulsion? The torque of the motor drops monotonically with increasing speed, and the relationship appears to be approximately linear over a wide range (Fig. 3). These results do not favour a mechanism by which the motor runs at constant power: in that case the torque would be inversely proportional to speed, and the fits would be hyperbolic. This conclusion is reinforced by two additional observations, namely, that the torque generated by a tethered cell is approximately constant for speeds from ~10 Hz to stall (ref. 11 and M.M., H.C.B., in preparation), and that the speed observed with hooks approximates the speed observed with flagellar bundles. The frictional drag coefficient of a hook is very much smaller than that of a filament in a bundle. Therefore, the intercepts of the linear fits with the axes of Fig. 3 are confirmed experimentally.

In the framework of thermodynamics, one can treat the flagellar motor as a system coupling two flows: the flux of protons and the rotation of the rotor. If the system operates close to thermodynamic equilibrium, then the flows are linear functions of the pair of conjugate driving forces, namely, the protonmotive force and the torque acting on the rotor¹⁷. When the protonmotive force is held constant, as it was in the measurements of Fig. 3, torque should decrease linearly with speed. Chemical processes occur close to equilibrium when the difference in the free energies of reactants and products (dissipated as heat) is small compared to kT , where k is the Boltzmann constant and T is the absolute temperature. The observed linear relationship between torque and speed appears to extend to the point where the motor generates essentially no torque and, thus, performs no mechanical work. At this speed, all the free energy available per proton (~8 kT) is dissipated by processes internal to the motor. The motor could still operate in the linear regime if the

torque-generating cycle associated with the transfer of one proton involved a series of many reactions, each of which entailed only a small free energy loss.

A mechanism that has this feature has been proposed by Lauger¹⁸, where protons are translocated from the external medium into the cytoplasm along binding sites at the intersections between rows of ligands on the faces of the M and S rings. The two sets of rows are tilted with respect to each other, so proton movement is coupled to motor rotation. Lauger discussed the operation of this device at low speeds and suggested that the torque should be independent of speed below 10 Hz. At the much higher speeds observed in swimming cells, finite proton transfer rates might lead to considerable dissipation of free energy conduction along the ligand chain and thus limit the torque. If each row contains 10 or more sites, the torque might still vary linearly with speed.

In a model proposed by Berg and Khan¹⁹, a single torque-generating step uses all the electrochemical energy of a proton and stores it as elastic energy in a spring. This process becomes rate-limiting at high speeds and accounts for all the internal dissipation. A preliminary analysis of this mechanism at steady state predicts a nonlinear dependence of torque on speed that is inconsistent with the data of Fig. 3. However, the model can be altered successfully by assuming that protons reach the rotor by stepping along a series of sites in a membrane channel, provided that the latter process is rate-limiting at high speeds.

An approximately linear torque-speed relationship can be obtained from mechanisms that work far from thermodynamic equilibrium, but only explicit construction. An example is the model proposed by Oosawa and Hayashi²⁰, in which proton transfer and rotation are loosely coupled. Measurements of torque at high speed place strict requirements on all models for the bacterial motor and deserve more attention.

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1. Berg, H. C. & Anderson, R. A. *Nature* **245**, 380–382 (1973).
2. Berg, H. C., Manson, M. D. & Conley, M. P. *Symp. Soc. exp. Biol.* **35**, 1–31 (1982).
3. Macnab, R. M. & Aizawa, S.-I. *A. Rev. Biophys. Bioeng.* **13**, 51–83 (1984).
4. Berg, H. C. *Random Walks in Biology*, 78–79 (Princeton University Press, New Jersey, 1983).
5. Reichert, K. *Zentr. Bakt. Parasitenkunde Ab. 1 Orig.* **51**, 14–94 (1909).
6. Berg, H. C. *A. Rev. Biophys. Bioeng.* **4**, 119–136 (1975).
7. Lowe, G. thesis Calif. Inst. Technol. (1987).
8. Berg, H. C. & Turner, L. *Nature* **278**, 349–351 (1979).
9. Schneider, W. R. & Doetsch, R. N. *J. Bact.* **117**, 696–701 (1974).
10. Khan, S. & Berg, H. C. *Cell* **32**, 913–919 (1983).
11. Manson, M. D., Tedesco, P. M. & Berg, H. C. *J. molec. Biol.* **138**, 541–561 (1980).
12. Banks, G., Schaefer, D. W. & Alpert, S. S. *Biophys. J.* **15**, 253–261 (1975).
13. Maeda, K., Imae, Y., Shioi, J.-I. & Oosawa, F. *J. Bact.* **127**, 1039–1046 (1976).
14. Miller, J. B. & Koshland, D. E., Jr *J. molec. Biol.* **111**, 183–201 (1977).
15. Ishihara, A., Segall, J. E., Block, S. M. & Berg, H. C. *J. Bact.* **155**, 228–237 (1983).
16. Armstrong, J. B., Adler, J. & Dahl, M. M. *J. Bact.* **93**, 390–398 (1967).
17. Prigogine, I. *Introduction to Thermodynamics of Irreversible Processes* 2nd edn (Interscience, New York, 1961).
18. Lauger, P. *Nature* **268**, 360–362 (1977).
19. Berg, H. C. & Khan, S. in *Motility and Recognition in Cell Biology* (eds Sund, H. & Veeger, C.) 486–497 (de Gruyter, Berlin, 1983).
20. Oosawa, F. & Hayashi, S. *J. Phys. Soc. Japan* **52**, 4019–4028 (1983).
21. Angelini, F., Ascoli, C., Frediani, C. & Petracchi, D. *Biophys. J.* **50**, 929–936 (1986).
22. Berg, H. C. & Block, S. M. *J. gen. Microbiol.* **130**, 2915–2920 (1984).
23. Segall, J. E., Ishihara, A. & Berg, H. C. *J. Bact.* **161**, 51–65 (1985).
24. Berg, H. C. *Adv. opt. Elect. Microsc.* **7**, 1–15 (1978).
25. Reinsch, C. H. *Numer. Math.* **10**, 177–183 (1967).
26. Van der Drift, C., Duiverman, J., Bexkens, H. & Krijnen, A. *J. Bact.* **124**, 1142–1147 (1975).
27. Berg, H. C. & Brown, D. A. *Nature* **239**, 500–504 (1972).
28. Tirado, M. M. & de la Torre, J. G. *J. chem. Phys.* **71**, 2581–2587 (1979).
29. Tirado, M. M. & de la Torre, J. G. *J. chem. Phys.* **73**, 1986–1993 (1980).
30. Heimbrook, M. E., Wang, W. L. L. & Campbell, G. *Abstracts of the Annual Meeting of the American Society for Microbiology*, 1986, 240 (American Society for Microbiology, Washington DC, 1986).
31. Berg, H. C. in *Cell Motility* Vol. 3C (eds Goldman, R., Pollard, T. & Rosenbaum, J.) 47–56 (Cold Spring Harbor Laboratory, New York, 1976).

Mammalian and bacterial sugar transport proteins are homologous

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The uptake of a sugar across the boundary membrane is a primary event in the nutrition of most cells, but the hydrophobic nature of the transport proteins involved makes them difficult to characterize. Their amino-acid sequences can, however, be determined by cloning and sequencing the corresponding gene (or complementary DNA). We have determined the sequences of the arabinose- H^+ and xylose- H^+ membrane transport proteins of *Escherichia coli*. They are homologous with each other and, unexpectedly, with the glucose transporters of human hepatoma¹ and rat brain² cells. All four

proteins share similarities with the *E. coli* citrate transporter¹⁸. Comparisons of their sequences and hydropathic profiles yield insights into their structure, functionally important residues and possible evolutionary relationships. There is little apparent homology with the lactose- H^+ (LacY)³ or melibiose- Na^+ (MelB)⁴ transport proteins of *E. coli*.

In the bacterium *Escherichia coli* there is one H^+ -linked transport system for arabinose encoded by the gene *araE*⁵, and another for xylose encoded by *xylE*⁶ (with gene products AraE and XylE). The linkage of sugar and H^+ translocation enables energization of nutrient uptake by the transmembrane electrochemical proton gradient^{7,8}.

We have isolated and sequenced the *araE* and *xylE* genes, and deduced the amino-acid sequence of each protein (Fig. 1). AraE contains 472 amino acids (relative molecular mass (M_r) 51,683), and XylE contains 491 amino acids (M_r 53,607). Both are typical membrane proteins: they are very hydrophobic (about 67% nonpolar residues); their hydrophobic and hydrophilic regions are arranged alternately (Fig. 1; see also ref. 9); and their calculated M_r values are substantially higher than the values of ~36,000 for AraE⁸ and 39,000 for XylE⁸ measured by SDS-polyacrylamide gel electrophoresis.

The amino-acid sequence of the glucose transport protein, which does not translocate a cation, from a human hepatoma cell line (HepG2) was recently determined¹. The reading frame

Fig. 1 Aligned sequences of the *E. coli* xylose- H^+ , arabinose- H^+ and citrate transporters with the human hepatoma glucose transporter. The sequences of the glucose and citrate transport proteins were taken from refs 1 and 18, respectively. Residues are boxed in the XylE, glucose and AraE transporters if they occur more than once at an aligned position in the three sugar transporter sequences. They are only boxed in the citrate transporter if they also occur in the aligned positions of two or three of the sugar transporters. The predicted membrane-spanning regions of the glucose transporter¹ are underlined. X, XylE; A, AraE; C, citrate transporter; G, glucose transporter.

Methods. The *araE* gene is located at 61 min on the linkage map close to the *lysA* gene⁸. A restriction map of a λ plac Mu phage inserted into the *araE* gene established its precise position by comparison with an overlapping restriction map of the *lysA* region³⁶. This enabled a 2.8-kilobase (kb) DNA fragment containing the intact *araE* gene and promoter region to be cloned from a specialized λ transducing phage³⁷ into the multicopy vector pBR322. The resultant plasmid, pMM25, conferred arabinose- H^+ transport activity on appropriate recipient *E. coli* strains negative for *araE*. The *xylE* gene is located at 91.4 min on the linkage map between the *pgi* and *malB* genes⁶. A restriction map of a λ plac Mu phage inserted into the *xylE* gene established its precise position by comparison with an overlapping restriction map of the *malB* region³⁸. This enabled a 2.8-kb DNA fragment containing the intact *xylE* gene to be cloned from a λ (*xylE*) Φ (*malK-lacZ*) phage into the multicopy vector pBR328. The resultant plasmid, pEJ3, conferred xylose- H^+ transport activity on appropriate recipient *E. coli* strains deleted for *xylE*. The complete DNA sequence of each 2.8-kb fragment and criteria for identifying the reading frame will be described elsewhere.

```

X  - - - - - MNTQYNSSYIFSLTLVATLGGGLL-FGGYDPAIVISSTVESLNTVPVAPQ- 46
G  - - - - - MEPSKSKKLTRRL-M-LAVGGAVLGLSLQPGYVNWGVINAPQKVITEE-FYNQWTW 48
A  MVTINTESALTPRSLRLDRTRRMNMFFVSVAANAVALGLL-FGLDGLGVLAAGALPFTDTHFVLTLS- 58
C  - - - - - MTQQPSRAGTFGAILRVTSGNLFLEQDFDFL-FGIFYATYIAKTFPPAESEFAALM- 53

X  - - NLSBSAANSLU--GFCVASALIGCIITGGALGGYCSNRRFGRGRDSSLKIAAVLFFPISGVG 101
G  VHRVYGESEILPTTLTTLWLSLSVAIFSVGGMIGSFSVGLFVNRRFGRGRNSSLMMNMLLAVFVLSV 109
A  - - RLQRE-- - - - - - WVVSMMHLCAGLALFNGLSFRLLGRKYSLLMACAILEFVLGSIQ 105
C  - - - - - LTFAVFVGSGFLMRPITDAVVLGDAYIDRIIGRRKGLMITLAIMCGCTLL 99

X  SIAWPELQFTTSINPDNTVPVYLACYVVPFVYI-RIIGGIGVGLASMLSPMVIARLAPAHIRG 161
G  MGRSKLGLK-- - - - - - SFBMLILGRFPIIGVYCGLLTTCGFVPMYVGEVSEFTAFRIG 154
A  SIAFAT-- - - - - - SVBMLIIAARVVLGLTAVGIGIASYTAPLYLSEBMASENVRIG 147
C  IALVPGYQTIGLLAPVLLV-- - - - - - VGRLLQGFSAQVELGGVSVVYLSIATPGNKKG 149

X  KLVSPFNQPAIFQDQVYCVNYFIARSQDASWLNNTDGRYMFASECTPAPATLFLMDEYTVFP 222
G  ALGTLHQUGIVVGLTLLA-- - - - - - QVFGLDSSIMGNKDLWFLDLSIIFTPAPATLQCTVLPFC 209
A  KMISSMYQLMVTTLGLVLA-- - - - - - HLDSTAFSYSGNWRANMLGVLALFAVLLEICVVP 200
C  FYTSWQASASQQAIVVVAALIGYGLNVTGLGHDEISEWGRIRIPFFIGCMIIIPVIFVRRSLQ 210

X  SPRFVLMSS-RGKQEQAEGLDRKIMGNLTALP-QAVQRERKHSLSDHGRKKTGG-- - - - RLLMFG 274
G  SPRFVLLINRNEENRAKSVLLKGRGRADYTH-GLDQGMKGRSRQMMREKKVITILELFBSPAY 268
A  SPRFVLLAE-KGRHIEAKSVLLKGRGRADYTH-GLDQGMKGRSRQMMREKKVITILELFBSPAY 268
C  TEAF-- - - - - - QRKHRPDTREIFTTIA-- - - - - - KNW 234

X  VGVIVIGVMSLSIFQQQFVGINVVLVYAFPEVFKTLGAS-NDIALLLQ-PIIIVGVINLTFFVLAI 333
G  RQPPLLIIVVLLQLSLQQLSGLINAVFYISTSTPEKAGV-- - - - RQPPVYATLIGSGIVNTAFITV 325
A  BRAVFLGLMLLQAMQQLTGMNIIIMVYAFPEVFKTLGAGFTTTRERQMI-ANLVLVGLTTFMFAFI 315
C  R-I-TAGTLVAMTT-TTFYFITVXTFTYGRVTLNL-SARDSLVVMTLVGISNFIWLP 292

X  MFDVDFKFGRRKPLQIIGALGMAIOMFSPDGFATFYT-- - - - QAP-- - - - GIVALLSMLFYV 386
G  FVVERAGRRRTLHLIGLAGMA-GCAILMTIAALLLELPWM-- - - - SYLSIVAIIFGFVAF 382
A  FVVDKACGRKPAKLGFSVMALQGLTLVLYCYC-LMQPDNGTASSGLSWLSVGMTMNCIAGY 375
C  AISDRICRRPVLMTGITLLALVLTLPVMMNLTAAPD-- - - - - - FTRMTLVLLWFS 344

X  WIPVVCVVLVLSBLFSPNAIRGKALATAVIA-- - - - AQLANVYFVSWPFPMDKNSWLVA 445
G  GPPIPFVFIIVALELFSQGRFP-- - - - AATAVAGFSNNTSNFTVGMCFQY-- - - - VEQ 434
A  AAPVIVVILCSSEIQP-- - - - LKCRDFGLTCTTTNWVSNMIIIGATFLTL-- - - - L 428
C  YNGAMVAALTGVNPFVYVRTVGFSLAPSLATAIIFGGLTPAISTALVQ-- - - - LTGDKSS 400

X  WITYG-CMGVLAALFMWKFPVPRFKKRLDBRLBALWEPETKKTQQTATL-- - - - - - 491
G  IIFV-VMLVLFVFIFFPKVPRFKKRLDBRLBALWEPETKKTQQTATL-- - - - - - 492
A  VLYN-ALNIAFVFGITFFVLRKLMAGEKLRNIGV-- - - - - - 472
C  WLMCAACGIAATTMLFARLSSGYQVWKNL-- - - - - - 431

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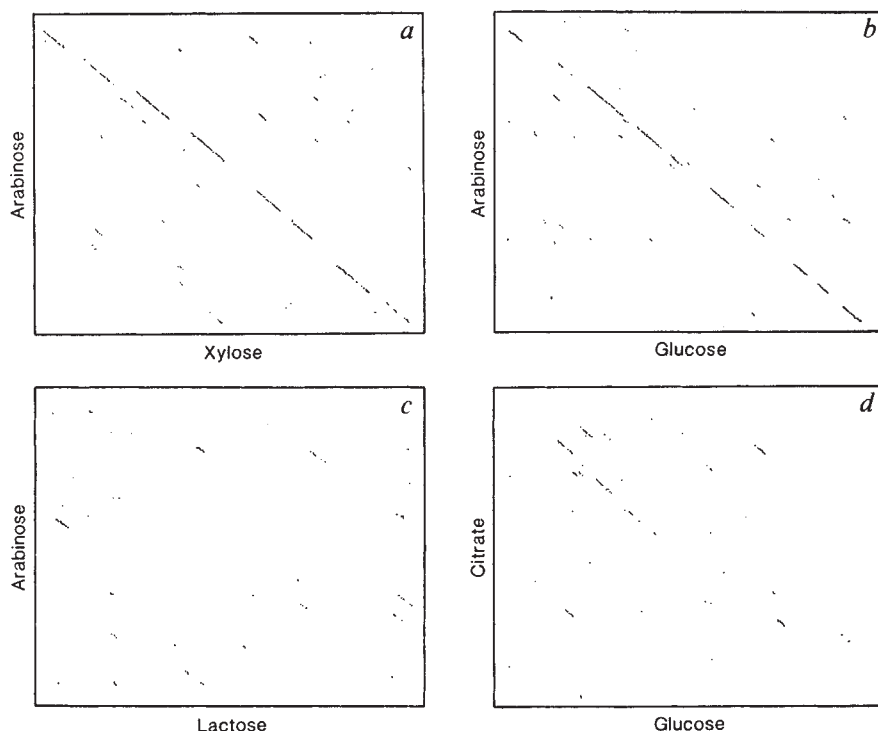



Fig. 2 Comparisons of the sequences of transport proteins using Diagon plots¹⁰. *a*, The arabinose-H⁺ and xylose-H⁺ transport proteins of *E. coli*; *b*, the arabinose-H⁺ transport protein of *E. coli* and glucose transport protein of human hepatoma cells; *c*, the arabinose-H⁺ and lactose-H⁺ transport proteins of *E. coli*; *d*, the citrate transport protein of *E. coli* and the glucose transport protein of human hepatoma cells. The comparisons used a 21-residue segment and generated a dot if the score exceeded 240 (ref. 10).

was confirmed by the identity of parts of its sequence with peptides derived from the erythrocyte glucose transporter¹. Its amino-acid composition was similar to that of the AraE and XylE proteins with 492 amino acids (M_r 54,117)¹. The sequence of the glucose transporter from rat brain cells, identified and cloned using similar techniques, was virtually identical, having 492 amino acids (M_r 56,133)².

The sugar-transporter sequences were compared using the Diagon algorithm of Staden¹⁰. Several homologous regions were revealed (Fig. 2*a, b*) in both hydrophobic and hydrophilic regions (Fig. 1). Furthermore, their hydropathic profiles^{9,11} were similar (Fig. 3); there was an hydrophilic segment, 50–65 residues long, located approximately at the mid-points of each of the sequences (Fig. 3), with some similar hydrophilic segments either side marked X in Fig. 3, and a similar number (12) of hydrophobic segments (Fig. 3).

Diagon plots drawn at the same and less stringent levels of discrimination did not reveal such homologies between XylE, AraE or the glucose transporter and the lactose³ or melibiose⁴ transporters of *E. coli* (one example is shown in Fig. 2*c*). It should be noted that the scoring matrix used by the Diagon algorithm was not devised for membrane proteins.

The sequence of the passive glucose transporter¹ is aligned with the sequences of the active arabinose and xylose transport proteins in Fig. 1. Seventy-seven residues are conserved in all three proteins (the glucose transporter has 131 identities with AraE and 142 identities with XylE; AraE and XylE have 141 residues in common). There are additional conservative substitutions¹² throughout the sequences. Consequently nearly 40% of residues can be regarded as homologous, a level sufficient to expect similar secondary and tertiary structures for all three proteins. Like many integral membrane proteins¹³ these transporters lack the N-terminal signal sequences typically required for insertion of proteins through membranes¹⁴. The attachment site for the carbohydrate chain, Asn 45 of the hepatoma cell glucose transporter^{1,15}, is not conserved in the XylE or AraE proteins (Fig. 1).

The citrate-H⁺ transporter¹⁶ of *E. coli* has 431 amino acids (M_r 46,979)^{17,18} and an apparent M_r of 35,000¹⁹ (determined by SDS-polyacrylamide gel electrophoresis. Diagon comparisons

revealed little homology with AraE or XylE, but some homology with the glucose transporter (Fig. 2*d*). However, all four proteins had similar hydropathic profiles (Fig. 3) with 12 membrane-spanning regions predicted by the Eisenberg algorithm¹¹ (Fig. 1, Fig. 3). This enabled us to align the patterns of conserved amino acids in all four transporters, particularly in the regions 30–49, 60–101, 121–149, 257–302 (Fig. 1). These patterns may be coincidental²⁰, but, more probably, they indicate that those residues boxed in the citrate transporter (Fig. 1) are critical for the common transport function of all the proteins.

It is interesting to locate certain conserved amino acids, in view of previous suggestions as to their roles in transport. For example, a glutamate (or aspartate) residue is responsible for H⁺-translocation by subunit c in the F₀ moiety of H⁺-ATPase²¹, and Glu 325 is implicated in H⁺-translocation by LacY²², so the conservation of such residues at positions corresponding to 153, 337, 397 and 472 of XylE (Fig. 1) identifies putative H⁺-translocating residues in these proteins. The mammalian transporters are not thought to translocate cations, however²³.

There is no conservation of histidines and cysteines (Fig. 1), residues implicated in the transport function of LacY^{24–27}. Hence they are unlikely to have a common role in these proteins. In all three sugar transporters substrate(s) protect a thiol group against reaction with *N*-ethylmaleimide^{8,28,29}, a phenomenon well established in LacY^{23,24,30}. As all cysteines in AraE occur between residues 343–400, at least a part of this region of this protein is adjacent to a substrate binding site.

A compelling conserved feature is the motif RXGRR, which is duplicated in each of the four proteins (Fig. 1; Fig. 3 marked X; R may be replaced by K), and occurs in a similar form in LacY and MelB (Fig. 3). It is predicted by the Robson algorithm³¹ to form a β -bend in six of its eight locations. This may link two helices in a defined topology stabilized by charge-charge interactions with head groups of the lipids. Its sequence also resembles peptides recognized by mammalian cyclic AMP protein kinase^{32,33}.

The central hydrophilic regions of all four proteins are predicted by the Robson algorithm³¹ to be α -helices, which correlates with the observation that 20% of the homologous erythrocyte glucose transporter forms non-membrane α -helix³⁴. There are

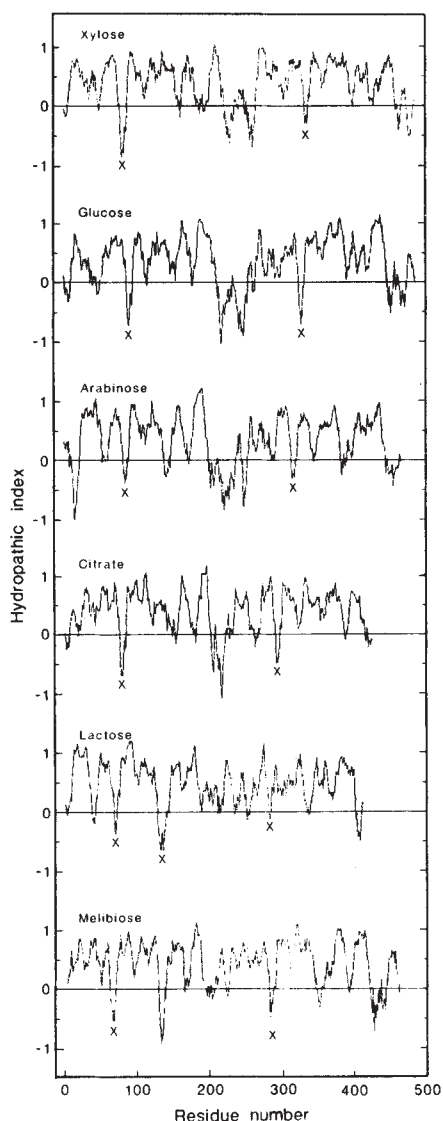


Fig. 3 Hydropathic profiles of transport proteins. Hydropathy values⁹ for a window of nine amino-acid residues were averaged and plotted according to the position of the middle residue along the length of each sequence. The sequences of the indicated proteins were taken from Fig. 1 and refs 1, 18, 3 and 4. X indicates the positions of the -RXGRR- motif in each protein.

indications, such as the duplicated RXGRR motif, that the N-terminal and C-terminal halves are arranged symmetrically about this region, with six corresponding membrane α -helices on each side. An internal gene duplication event²⁰ is thus implied in an ancestral transporter, which then evolved to the present proteins.

The similarity between the sugar transporters from such divergent organisms as bacteria and mammals is probably too great to have arisen by convergent evolution. Gene transfer from eukaryotes to their prokaryotic symbionts remains an explanation: an example of this process has recently been described³⁵. But we think it more likely that the homologies reflect functionally important parts of an ancient sugar transporter present in organisms before their divergence into prokaryotes and eukaryotes. If this is the case, homologous sugar transporters may also be found in plants, unicellular eukaryotes and other organisms. Whatever its origin, the similarity of these transport proteins suggests that a combination of biochemical, immunological and genetic techniques can now be used to exploit the advantages of working with prokaryotes to illuminate transport processes in higher organisms.

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1. Mueckler, M. *et al. Science* **229**, 941-945 (1985).
2. Birnbaum, M. J., Haspel, H. C. & Rosen, O. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5784-5788 (1986).
3. Buchel, D. E., Gronenborn, B. & Muller-Hill, B. *Nature* **283**, 541-545 (1980).
4. Yazuy, H. *et al. J. biol. Chem.* **259**, 4320-4326 (1984).
5. Daruwalla, K. R., Paxton, A. T. & Henderson, P. J. F. *Biochem. J.* **200**, 611-627 (1981).
6. Davis, E. O., Jones-Mortimer, M. C. & Henderson, P. J. F. *J. biol. Chem.* **259**, 1520-1525 (1984).
7. Mitchell, P. J. *Bioenerg.* **4**, 63-91 (1973).
8. Henderson, P. J. F. & Macpherson, A. J. S. *Meth. Enzym.* **125**, 387-429 (1986).
9. Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105-132 (1982).
10. Staden, R. *Nucleic Acids Res.* **10**, 2951-2961 (1982).
11. Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R. J. *J. molec. Biol.* **179**, 125-142 (1984).
12. Dayhoff, M. O., Schwartz, R. M. & Orcutt, B. C. in *Atlas of Protein Sequence and Structure* Vol. 5, Suppl. 3 (ed. Dayhoff, M. O.) 345-352 (National Biomedical Research Foundation, Silver Spring, 1978).
13. Walker, J. E. & Fearnley, I. M. in *Techniques for the Analysis of Membrane Proteins* (eds Ragan, C. I. & Cherry, P.) 235-273 (Chapman & Hall, New York, 1986).
14. Von Heijne, G. *J. molec. Biol.* **184**, 99-105 (1985).
15. Mueckler, M. & Lodish, H. F. *Cell* **44**, 629-637 (1986).
16. Reynolds, C. H. & Silver, S. *J. Bact.* **156**, 1019-1024 (1983).
17. Ishiguro, N. & Sato, G. *J. Bact.* **164**, 977-982 (1985).
18. Sasatsu, M., Misra, T. K., Chu, L., Laddaga, R. & Silver, S. *J. Bact.* **164**, 983-993 (1985).
19. Hirato, T., Shinagawa, M., Ishiguro, N. & Sato, G. *J. Bact.* **160**, 421-426 (1984).
20. Doolittle, R. F. *Science* **214**, 149-159 (1981).
21. Walker, J. E., Saraste, M. & Gay, N. J. *Biochim. biophys. Acta* **768**, 164-200 (1984).
22. Carrasco, N., Antes, L. M., Poonian, M. S. & Kaback, H. R. *Biochemistry* **25**, 4486-4488 (1986).
23. Wright, J. K., Seckler, R. & Overath, P. A. *Rev. Biochem.* **55**, 225-248 (1986).
24. Kaback, H. R. *Ann. N.Y. Acad. Sci.* **456**, 291-304 (1986).
25. Sarkar, H. K. *et al. Meth. Enzym.* **125**, 214-230 (1986).
26. West, I. C. *Biochem. Soc. Trans.* **8**, 706-707 (1980).
27. Konings, W. N. & Robillard, G. T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5480-5484 (1982).
28. Batt, E. R., Abbott, R. E. & Schachter, D. *J. biol. Chem.* **251**, 7184-7190 (1976).
29. Deziel, M. R., Jung, C. Y. & Rothstein, A. *Biochim. biophys. Acta* **819**, 83-92 (1985).
30. Beyreuther, K., Bieseler, B., Ehring, R. & Muller-Hill, B. in *Methods in Protein Sequence Analysis* (ed. Elzina, M.) 132-148 (Humana, Clifton, 1981).
31. Garnier, J., Osguthorpe, D. J. & Robson, B. *J. molec. Biol.* **120**, 97-120 (1978).
32. Feramisco, J. R., Glass, D. B. & Krebs, E. G. *J. biol. Chem.* **255**, 4240-4245 (1980).
33. Cheng, H. C. *et al. J. biol. Chem.* **261**, 989-992 (1986).
34. Chin, J. J., Jung, E. K. Y. & Jung, C. Y. *J. biol. Chem.* **261**, 7101-7104 (1986).
35. Carlson, T. A. & Chelm, B. K. *Nature* **322**, 568-570 (1986).
36. Stragier, P. & Patte, J. C. *J. molec. Biol.* **168**, 333-350 (1983).
37. Macpherson, A. J. S., Jones-Mortimer, M. C. & Henderson, P. J. F. *Biochem. J.* **196**, 269-283 (1981).
38. Marchal, C., Greenblatt, J. & Hofnung, M. *J. Bact.* **136**, 1109-1119 (1978).

Molecular dynamics studied by analysis of the X-ray diffuse scattering from lysozyme crystals

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It is now well established that the biological activity of proteins is related not only to their mean molecular structure, but also to their intramolecular mobility¹⁻⁴. Nearly all techniques sensitive to dynamics have given evidence for intramolecular mobility in proteins: NMR^{5,6}, ESR⁷, Raman spectroscopy^{8,9}, fluorescence quenching¹⁰, Mössbauer spectroscopy¹¹, neutron scattering¹², measurements of elastic constants¹³ and hydrogen-deuterium exchange¹⁴. The dynamics of proteins has also been approached by theoretical calculations^{15,16}. We report here investigations of the atomic and molecular displacements in hen egg-white lysozyme crystals using a new technique. This technique, based on the X-ray diffuse scattering analysis (scattering out of the Bragg reflections),

can yield information on the atomic displacements, provided that they are correlated. Rigid-body molecular displacements, correlated along short rows of aligned molecules in two perpendicular directions, have been detected and analysed (mean square amplitude of the order of $5 \times 10^{-4} \text{ nm}^2$). This technique can be also applied to the detection and analysis of intramolecular displacements.

Molecular mobility can alter the periodic order of protein crystals, giving imperfect crystals. The concepts of X-ray scattering by imperfect crystals have been clearly explained by Guiner¹⁷ and Cowley¹⁸. Basically, the effect of imperfections is to split the scattered intensity into two components I_B and I_D . The term I_B corresponds to the set of Bragg reflections; Debye¹⁹ has shown that for small amplitude and harmonic displacements I_B can be derived from the expression which is valid for a perfect crystal by multiplying each atomic scattering factor by the factor $\exp(-B|S|^2/4)$ where S is the scattering vector and B the temperature factor. For each atom j , $B = 8\pi^2 \langle u_j^2 \rangle$ where u_j^i is the projection of the displacement of atom j along S . The term I_D is the diffuse scattering intensity. In contrast to I_B , which depends on the mean value of the scattering function per unit-cell, I_D depends on the local deviations of this function from its mean value and on its spatial correlations; it is spread all over reciprocal space and unlike I_B is not restricted to the reciprocal lattice nodes. Thus, even in the case of long range correlations, I_D will be ≥ 100 times weaker than I_B . Consequently, its observation requires high-intensity beams, long exposures and a low background. Analyses of B -factors and comparison between crystalline structures (which are determined from I_B) has provided valuable information concerning the flexibility of various proteins²⁰⁻²³.

But more precise B -factor analyses²⁴ remain limited by various factors. Firstly the $\langle u^2 \rangle$ determinations hold only for small harmonic displacements and values of $\langle u^2 \rangle$ are considered as isotropic for proteins (except in refs 25 and 26). The most important limitation stems from the fact that the thermal factors are individual factors, summing up all the types of displacements, and thus no correlation between the atomic displacements can be derived from their values. The correlation information can be obtained precisely by the diffuse scattering study, which gives access to the deviations from the ideal mean crystalline structure. The existence of diffuse scattering was predicted as early as 1923 by Waller²⁷, and has been widely and successfully used in physical and chemical crystallography. This technique has only been used once for proteins by Phillips *et al.*, who derived a model of filament motion for tropomyosin²⁸. Our aim is to extend the use of diffuse scattering analysis to biological macromolecular compounds.

We have tested the feasibility of diffuse scattering studies of proteins on hen egg-white lysozyme crystals. The dynamics of this enzyme has been extensively studied by various techniques^{9,13,14,16,29-31} and by analysis of thermal factors^{23,32}. This work is restricted to the orthorhombic form of lysozyme which is stable at physiological temperatures. The structure of this crystalline form has been determined at 0.2 nm resolution by Berthou *et al.*³³. The data collection of diffuse scattering has been performed at the Laboratoire d'Utilisation du Rayonnement Electromagnétique (LURE) using synchrotron radiation. Because of the much longer exposure times than for recording Bragg reflections (10^2 – 10^4 times), only a set of 'still' photographs has been obtained.

On the film, the diffuse intensity can be observed along rows, with a modulated intensity, and is in fact, in the reciprocal space, mainly located along two families of planes: $(100)^*$ (perpendicular to a^*) and $(001)^*$ (perpendicular to c^*). Other tiny diffuse features are found along the reciprocal planes $(101)^*$ and $(10\bar{1})^*$, but we will not consider these two poorly defined features.

We can at once discard the hypothesis that attributes the diffuse scattering to radiation damage effects, because fresh

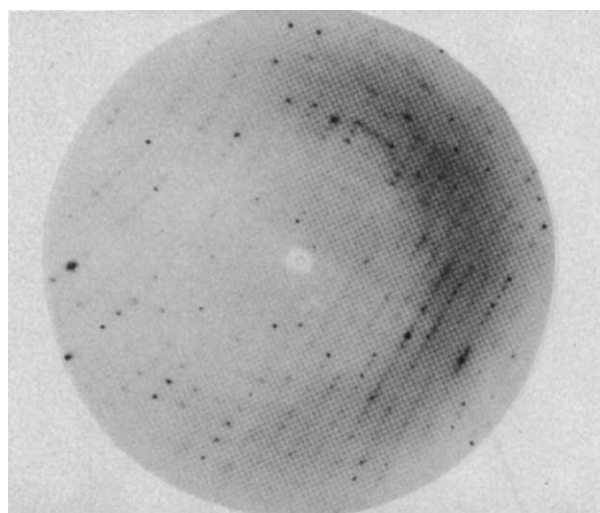


Fig. 1 Typical example of an X-ray scattering pattern from hen egg-white lysozyme crystals (form B). The unit cell is orthorhombic (symmetry $P2_12_12_1$) with the parameters: $a = 5.64 \text{ nm}$, $b = 7.38 \text{ nm}$, $c = 3.05 \text{ nm}$. The crystals were grown at 40°C from a 5% protein solution buffered at pH 4.7 with 2.0 M acetate to which a 10% NaCl solution was added. The data have been obtained using the synchrotron radiation of LURE on station D16 (Paris-Sud University). A single bent germanium crystal allows work with a low background beam ($\lambda = 0.161 \text{ nm}$). The photographs have been obtained using a STOE Explorer Camera in fixed geometry (crystal-film distance 75 mm) with exposure times as long as 30 min whereas the recording of the Bragg reflections needed only a few seconds; the diameter of the collimator was 0.5 mm. About 30 still patterns, at a resolution of 0.22 nm, have been obtained for various crystal orientations, precession angle values and positions during the 'precession motion'. This set of patterns, which includes all the major zones, is normally sufficient to exclude the possibility of having missed any major diffuse intensity. Four intensity components can be observed. The blackest spots are Bragg reflections. Each Bragg spot is surrounded by a diffuse scattering area which is the thermal diffuse scattering (TDS). When the Ewald sphere intersects a diffuse spot is recorded. The third component is the large diffuse ring (maximum around 3.0 nm^{-1}) which is due to the sample holder (capillary glass) and to the liquid water within and surrounding the crystal. The fourth component is the diffuse scattering which gives evidence of a disorder specific to the compound; it is mainly visible within the resolution range 0.8 nm^{-1} – 0.25 nm^{-1} . For this figure, the 'vertical' rows are the traces of reciprocal planes perpendicular to c^* and the 'horizontal' ones of planes perpendicular to a^* ; diffuse scattering is visible along both types of rows. The intensity asymmetry between the left and right sides of the film is due to the fact that this film was not perpendicular to the incident beam (it has been recorded with the precession angle 20°). The corresponding intensity corrections have been taken into account in our calculations.

crystals and old ones display exactly the same diffuse pattern. Because of the well-defined location of I_D , we have used a two-step analysis procedure: determination of the type of correlation involved in the atomic displacements (from I_D location) followed by determination of displacement parameters (from I_D intensity). This type of correlation is simply given by the Fourier transform of equidistant reciprocal planes (distance d^*) which lie in a periodic row (period $1/d^*$) perpendicular to the planes. In other words, the diffuse planes imply the existence in the crystal of periodic finite rows parallel to a and c , of period $|a|$ and $|c|$, which scatter the X-photons in a partially incoherent way because of atomic displacements. More precisely at any given instant, all the equivalent atoms (translation a or c related atoms) along a row are displaced from their mean position by the same vector u , the displacements being uncorrelated between different rows. The average length of these rows is given by the inverse of the thickness (FWMH) of the diffuse planes, (4 ± 1)

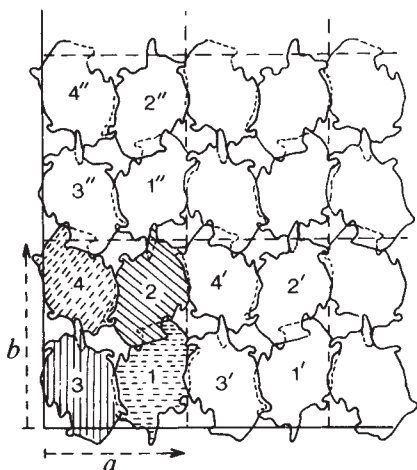


Fig. 2 Projection of the molecular envelopes onto the (a, b) plane. The unit cell contains four molecules: 1, 2, 3 and 4. The structure appears as alternate rows of molecules type (3-1-3'-1'...) and (4-2-4'-2'...) parallel to **a**, and (1-2-1'-2'...) and (3-4-3'-4'...) parallel to **b**. Only those parallel to **a** are real ones as their molecules have the same height along **c**, whereas the molecules forming the rows parallel to **b** have not. Along the **c** direction the structure can be also viewed as the juxtaposition of four types of rows (molecules 1-1..., 2-2..., 3-3..., 4-4...).

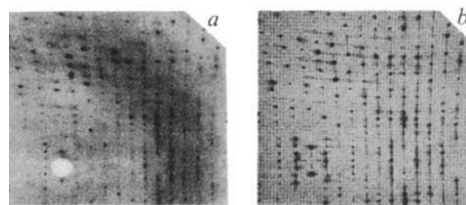


Fig. 3 Example of a comparison between experimental film (a) and simulation (b). The film intensity is simulated with the pixel density: 2,333 pixels nm², that is by 50,000 pixels. For each pixel, the intensity is the sum of three components: I_B , $I_{D(TDS)}$ and $I_{D(D)}$. I_B is zero except on the Bragg spots. $I_{D(TDS)}$ is calculated from the approximation of isotropic elastic constants: $I_{D(TDS)} = C \langle F \rangle^2 S^2 / q^2$, where q is the distance of the extremity of **S** to the closest reciprocal node, $\langle F \rangle$ is the mean structure factor and C is a factor depending on the elastic constants. The experimental resolution is taken into account for I_B and $I_{D(TDS)}$. The component $I_{D(D)}$ is calculated according to equations (2) and (3). $I_{D(TDS)}$ and $I_{D(D)}$ are empirically scaled to I_B (the quantitative measurements of $I_{D(D)}$ with respect to I_B , to determine the $\langle u^2 \rangle$ values, have required additional patterns obtained with various exposure times). Finally the total intensity is corrected for the polarization effect according to ref. 34 and for the asymmetry effect due to the precession angle. The graphic drawing is done using sixteen grey tones. The parameters which have been varied are mainly the nature of the molecules forming the unit content, the direction of the displacements and the superunit lengths. Our simulations are quite sensitive to all these parameters; nevertheless, in some cases, the distinction between various unit contents has required a careful comparison between the experimental films and the simulations. In this orientation, **a*** lies in a vertical plane and **c*** in a horizontal plane. The Bragg spot and TDS intensities are not in complete agreement because of a small disorientation between the experiment and the film. The intensity modulations along the diffuse planes are in agreement except in the centre of the simulation (up to 0.8-nm resolution) which is overestimated. This effect is due to the solvent within the crystal (about 33% in mass) which has not been taken into account in our calculations.

$|a|$ for the **a** rows and $(7 \pm 1) |c|$ for the **c** rows. We shall call 'unit' the set of atoms belonging to one period along a row (it may be part of molecule, molecule or group of molecules) and 'superunit' the whole atomic content corresponding to one row, that is, four units **a** aligned in the **a** direction (superunits **a**) or seven units **c** aligned in the **c** direction (superunits **c**). The atomic content forming the units and the corresponding displacements have been determined by comparison between film simulations and experimental diffuse intensities. One example of simulation is given in Fig. 3. Three terms have been included in the simulations: the Bragg intensity I_B , and two I_D terms, $I_{D(TDS)}$ and $I_{D(D)}$. $I_{D(TDS)}$, which is located around each Bragg reflection, is due to the propagation of collective modes in crystals (thermal diffuse scattering, TDS).

For independent displacements:

$$I_{D(D)} = \sum_n K_n (\langle |F_n|^2 \rangle - \langle F_n \rangle^2) \quad (1)$$

where $\sum_n$ sums over the various types of superunits, K_n is a factor which takes into account the total number of individual superunits and F_n is the scattering factor of the n th superunit, which may be written

$$F_n(\mathbf{S}) = \sum_p \left[\sum_{j=1}^{J_n} f_j(\mathbf{S}) \exp(-2i\pi \mathbf{S} \cdot (\mathbf{r}^j + \mathbf{u}_p^j + \mathbf{u}_n^j)) \right] \times \exp(-2i\pi \mathbf{S} \cdot \mathbf{p} d_n) \quad (2)$$

where $\sum_p$ sums over the units of superunit n which are characterized by the correlation periodicity d_n (**a** or **c**), and $\sum_j$ sums over the J_n atoms of the unit associated with superunit n ; $f_j(\mathbf{S})$ is the atomic scattering factor of atom j . The displacements $\mathbf{u}_p^j$ give rise to TDS and only the $\mathbf{u}_n^j$ which are correlated within a superunit are of interest. Satisfactory simulations have been obtained with small amplitude and harmonic rigid-body displacements, that is, with translations $\mathbf{u}_n$ of the whole superunit. In that case, (1) and (2) lead to the formula:

$$I_{D(D)} = K_n \langle F_n \rangle^2 (1 - \exp(-4\pi^2 \langle (\mathbf{S} \cdot \mathbf{u}_n)^2 \rangle)) \quad (3)$$

Our results can be summarized as follows. The displacement directions are parallel to **a** for superunits **a** and to **c** for superunits **c**, with a precision of $\pm 15^\circ$. With regard to the superunit contents,

the best agreement is reached assuming two types of superunits **a**, one for which the unit contains molecules 1 and 3 and the other molecules 2 and 4 (see Fig. 2). There are also two types of superunits **c**, one for which the unit contains molecules 1 and 2, and the other molecules 4 and 3'. The last parameter extracted from our films is the amplitude of the displacements, from the comparison of the Bragg spots and the diffuse scattering intensities. This can be calculated from (3) by introducing the resolution effects and the total number of each type of superunit. With these conditions we get $\langle u_a^2 \rangle = (5 \pm 3) \cdot 10^{-4} \text{ nm}^2$ for the **a**-superunits, and $\langle u_c^2 \rangle = (7 \pm 3) \cdot 10^{-4} \text{ nm}^2$ for the **c**-superunits.

Our results prove the existence of intermolecular rigid-body displacements which are correlated within short periodic rows of aligned molecules along **a** and **c**. A satisfactory aspect of the results is that the superunits have a physical meaning, namely that the molecules forming a superunit are sterically coupled. For instance, Fig. 4 shows that the coupling between molecules 1 and 3 in the **a**-direction is ensured by many contacts; in contrast, no contact between molecules 3 and 4 exists, which explains the absence of correlation in the **b** direction. Regarding superunits **c**, the coupling between molecules 1 and 2 (or 4 and 3') is also plausible, as there is an overlap of these molecules looking along **c** which implies coupled displacements. It is difficult to determine the origin of the correlated displacements. It seems unlikely that they arise from a hinge-bending mode about an axis located in the (**a**, **c**) plane²³ as the displacements would display a component in the **b** direction, which is not observed. A possible cause is a local disorder, involving one or more residues in contact with a neighbouring molecule, which would produce a displacement of the latter, all displacements being correlated within a superunit. Such phenomena occur perhaps at the intermolecular contact between residues Asp 101

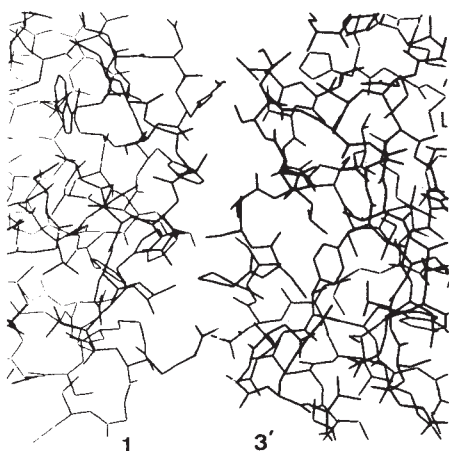


Fig. 4 Contacts between molecules 1 and 3'. The two molecular envelopes are complementary over an area of $\sim 0.28 \text{ nm}^2$. About 12 intermolecular contacts $< 0.4 \text{ nm}$ (involving residues Asn 113, Arg 114, Asp 119, Arg 125 of molecule 1 and Asn 65', Asp 66', Gly 67', Ser 81', Thr 89' of molecule 3') ensure a coupling between the molecules. The 15–20 well-localized water molecules in the intermolecular zone reinforce this coupling.

and Asn 103 of molecule 1 and Thr 47 and Asp 48 of molecule 2, which is characterized by distances between the mean atomic positions which are smaller than the van der Waals' distances.

We have shown that X-ray diffuse scattering can provide information not accessible by classical crystallography. In the case of hen egg-white lysozyme in the orthorhombic form, correlated rigid-body displacements of several molecules have been detected. It must be emphasized that, for this study, the diffuse scattering provides direct information only about rigid-body displacements of the molecules, that is intermolecular motions and intermolecular contacts. It is very likely that other protein crystals display diffuse scattering features due to intramolecular motions (such as domain motion). Their analysis can be performed in a similar way as the one reported here; our study must be considered as an initial study to develop a new approach to protein dynamics.

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- Huber, R. & Bennett, W. S. *Biopolymers* **22**, 261–279 (1983).
- Westhof, E. *et al. Nature* **311**, 123–126 (1984).
- Tainer, J. A. *et al. Nature* **312**, 127–134 (1984).
- Bennett, W. S. & Huber, R. *Crit. Rev. Biochem.* **15**, 291–384 (1984).
- Wüthrich, K. *Trends biochem. Sci.* **3**, 227–230 (1978).
- Fox, R. O., Evans, P. A. & Dobson, C. M. *Nature* **320**, 192–194 (1986).
- Christen, P. & Gehring, H. *Meth. biochem. Analysis* **28**, 151–174 (1982).
- Brown, K. G., Erfurth, S. C., Small, E. W. & Peticolas, W. L. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1467–1469 (1972).
- Genzel, L. *et al. Biopolymers* **15**, 219–225 (1976).
- Lakiwicz, J. R. & Weber, G. *Biochemistry* **12**, 4171–4179 (1973).
- Parak, F., Frolov, E. N., Mössbauer, R. L. & Goldanskii, V. I. *J. molec. Biol.* **145**, 825–833 (1981).
- Bartunik, H. D., Jollès, P., Berthou, J. & Dianoux, A. J. *Biopolymers* **21**, 43–50 (1982).
- Morozova, T. Y. & Morozov, V. N. *J. molec. Biol.* **157**, 173–179 (1982).
- Bentley, G. A. *et al. J. molec. Biol.* **170**, 243–247 (1983).
- Karplus, M. & McCammon, J. A. *CRC crit. Rev. Biochem.* **9**, 293–349 (1981).
- Levitt, M., Sander, C. & Stern, P. J. *J. molec. Biol.* **181**, 423–447 (1985).
- Guinier, A. *X-ray Diffraction* (Freeman, San Francisco 1963).
- Cowley, J. M. *Diffraction Physics* (North-Holland Amsterdam, 1975).
- Debye, P. *Annls Phys.* **43**, 49–95 (1914).
- Waller, J. *et al. Acta crystallogr.* **B38**, 1462–1472 (1982).
- Bennett, W. S. & Steitz, J. A. *J. molec. Biol.* **140**, 211–230 (1980).
- Frauenfelder, H., Petsko, G. A. & Tsernoglou, D. *Nature* **280**, 558–563 (1979).
- Sternberg, M. J. E., Grace, D. E. P. & Phillips, D. C. *J. molec. Biol.* **130**, 231–253 (1979).
- Schomaker, V. & Truebold, K. N. *Act. crystallogr.* **B24**, 63–76 (1968).
- Watenpugh, K. D., Sieker, L. C. & Jensen, L. H. J. *J. molec. Biol.* **138**, 615–633 (1980).

- Glover, I. *et al. Biopolymers* **22**, 293–304 (1983).
- Waller, I. *Z. Phys.* **17**, 398–408 (1923).
- Phillips, G. N., Fillers, J. P. & Cohen, C. *Biophys. J.* **32**, 485–502 (1980).
- McCammon, J. A., Gelin, B. R., Karplus, M. & Wolynes, P. G. *Nature* **262**, 325–326 (1976).
- Takizawa, T., Miyoshi, Y. & Saito, B. *Polymer J.* **6**, 85–93 (1974).
- Campbell, I. D., Dobson, C. M. & Williams, F. R. S. *Proc. R. Soc. A* **345**, 41–59 (1975).
- Artymiuk, P. J. *et al. Nature* **280**, 563–568 (1979).
- Berthou, J., Lifchitz, A., Artymiuk, P. & Jollès, P. *Proc. R. Soc. B* **217**, 471–489 (1983).
- Kahn, R. *et al. J. appl. crystallogr.* **15**, 330–337 (1982).

Erratum

The *Tetrahymena* ribozyme acts like an RNA restriction endonuclease

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In this article corrections for the last four paragraphs were not incorporated on page. The corrected version is printed here.

Sequence-specific endoribonucleases might have many of the same applications for the study of RNA, that DNA restriction endonucleases have for the study of DNA⁶. The pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be cleaved to fragments of a size more useful for study. The 4-nucleotide specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence; an RNA of random sequence would have an average of 1 cleavage site every 256 bases. The automatic end-labelling of one fragment during ribozyme cleavage is a practical advantage.

Development of the ribozyme as a useful tool for molecular biology has just begun. The cleavage efficiency of large RNA substrates needs to be improved, so that digestion is complete. The effects of denaturants such as urea and formamide must be further explored; they appear to increase the specificity of cleavage, and at the same time they should melt the secondary structure of the substrate to maximize the availability of target sequences. Finally, mutagenesis of the active site of the ribozyme must be continued to ascertain how many of the 256 possible tetranucleotide cleavage enzymes (of which we have investigated only four) work with acceptable efficiency and specificity.

Some protein ribonucleases cleave RNA substrates with high specificity by recognizing a combination of RNA structure and sequence; the structure is more important (for instance RNase III (ref. 28) and RNase M5 (ref. 29)). On the other hand known proteins that cleave single-stranded RNA substrates are only specific for mononucleotides or dinucleotides (for instance, RNase T₁ cleaves after guanines³⁰). Thus, the L-19 IVS RNA is considerably more sequence-specific for single-stranded RNA cleavage than any known protein ribonuclease.

RNA sequence recognition often involves recognition of RNA by RNA. Triplet codons in messenger RNA are recognized by anticodons in transfer RNA, and ribosome binding sites in prokaryotic mRNA are recognized using the Shine-Dalgarno interaction with 16S rRNA. It is easy to imagine uses for sequence-specific protein ribonucleases in pre-mRNA splicing and 3'-end generation, but there is no evidence for such enzymes. Instead, 5' splice sites in pre-mRNA are recognized using the U1 small nuclear RNA^{13,31} and the 3' end cleavage site of histone H3 pre-mRNA by U7 snRNA³²; much of the sequence discrimination is provided by complementary base-pairing. Perhaps it is difficult to construct an active site in a polypeptide to bind an unstructured stretch of nucleotides specifically. It may be possible that in certain reactions involving RNA substrates, RNA catalysis has not been superseded by protein catalysis for the simple reason that RNA does the job better.